

**Tuberculosis and genes of the IL12/IL23/IFN γ pathway:
Exploring functional significance of novel mutations in the
IL12p40 promoter**

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17 February 2014

Abstract

The aim of this work was to screen the IL12p40 gene promoter for association with TB disease. Initially a subcohort of children (TB cases and healthy controls) from a TB-endemic area was screened for DNA changes by the WAVE method. Thereafter, the entire paediatric cohort and a cohort of healthy adult controls were screened by Amplification Refractory Mutation System PCR. Functional testing was done by reporter assay and immunological phenotype was investigated by measurement of cytokines levels and cytokine receptor expression.

WAVE screening identified two heterozygous SNPs, -1523 A/G and -1564 C/T. Statistical analysis showed that -1523 A/G may be protective against TB disease ($p=0.02$). This possibility was supported by the location of -1523 A/G occurring within a GTATA sequence reported to bind nuclear proteins. Specific ARMS-PCR assays were then designed for screening of additional paediatric subjects and healthy adult controls for these SNPs. Analysis of the larger group, showed that -1564 C/T may contribute to susceptibility to TB disease ($p=0.03$). Exploring functional relevance, normal and mutant promoter fragments were PCR amplified, using uniquely adapted primers that included restriction sites corresponding to those in the multiple cloning site of an expression vector, facilitating cloning. A truncated promoter and one with essential regions deleted, were created as negative controls. These five promoter fragments were cloned into the expression vector and functional differences tested by reporter. No significant functional differences between variant and normal promoter fragments were observed. A predictive immune phenotype was investigated by measurement of IFN γ , TNF α and IL12p70 cytokine levels and IL12 β R1 receptor expression. While distinct patterns of cytokine responses were seen, these did not predict genotype. These results show that the IL12p40 gene promoter is highly conserved and sequence variants may be just one of many factors contributing to TB susceptibility.

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List of abbreviations

A	Adenine
AFB	Acid fast bacilli
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen presenting cell
ARMS-PCR	Amplification Refractory Mutation System PCR
ASP	Affected sibling pair
ATF	Activity transcription factor
BCG	Bacillus Calmette-Guérin
bp	Base pair
C	Cytosine
C/EBP	CCAAT/enhancer binding protein
CD	Cluster of differentiation
CGD	Chronic Granulomatous Disease
cm	Centimetre
CMV	Cytomegalovirus
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DBP	Vitamin-binding protein
DCE	Downstream core element
dCTP	Deoxycytidine triphosphate
DEAE	Diethylaminoethyl
DHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPE	Downstream promoter element
ds	Double stranded

EDA-ID	Ectodermal dysplasia with immunodeficiency
EDTA	Ethylenediaminetetraacetic
ELISA	Enzyme-linked immunosorbent assay
Ets2	Epithelium specific transcription factor 2
FCS	Foetal calf serum
G	Guanine
GAF	Gamma activating factor
GAS	Gamma activating sequences
GWAS	Genome wide association studies
H ₂ O ₂	Hydrogen peroxide
HGP	Human Genome Project
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HMA	Heteroduplex mutation analysis
HPLC	High-performance liquid chromatography
IFN γ	Interferon gamma
IFN γ R	Interferon gamma receptor
IKK	Inhibitor of NF κ B kinase
IL	Interleukin
IL-1 β	Interleukin 1 β
IL12	Interleukin 12
IL12R	Interleukin 12 receptor
IL12R β 1	Interleukin 12 receptor beta 1 chain
IL12R β 2	Interleukin 12 receptor beta 2 chain
IL12RE	Interleukin response element
IL-Ra	Interleukin 1 Ra
Inr	Initiator
IRAK	Interleukin 1 receptor-associated kinase

IRF1	Interferon regulatory factor 1
IRF8	Interferon regulatory factor 8
IRGM	Immunity related GTPase M
JAK	Janus activating kinases
JDP	Jun dimerization protein
KCl	Potassium chloride
kDa	Kilo dalton
LCH	Langerhans' Cell histiocytosis
LD	Linkage disequilibrium
LPS	Lipopolysaccharide
LTBI	Latent TB infection
<i>M. avium</i>	<i>Mycobacterium avium</i>
<i>M. tb</i>	<i>Mycobacterium tuberculosis</i>
MDGA	Mutation detection gel analysis
MDR	Multi drug resistant
Mg	Milligram
MHC	Major histocompatibility complex
ml	Millilitre
mRNA	Messenger RNA
MSMD	Mendelian Susceptibility to Mycobacterial Disease
NCBI	National Centre for Biotechnology Information
NEMO	NFkB essential modulator
NFkB	Nuclear factor kappa B
NK	Natural killer
NOS2A	Nitric oxide synthase 2A gene
NRAMP	Natural resistance-associated macrophage protein
NTM	Non-tuberculous Mycobacteria
OPD	O-phenylenediamine dihydrochloride

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
pmols	Picomoles
PPD	Purified protein derivative
PTB	Pulmonary tuberculosis
QC	Quality control
RCWMCH	Red Cross War Memorial Children's Hospital
rHu	Recombinant human
RIA	Radio immunoassay
RNA	Ribonucleic acid
ROX	6-carboxy-X-rhodamine
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SELDI	Surface-enhanced laser desorption/ionization
SH2	Src homology 2
SLC11A1	Solute carrier family 11, member 1
SNP	Single nucleotide polymorphism
SP1	Specificity protein 1
SSCP	Single stranded conformation polymorphism
STAT	Signal transducer and activator of transcription
T	Thymine
TB	Tuberculosis
TBM	Tuberculosis meningitis
TBP	TATAbox binding protein
TFIID	Transcription initiation factor IID
TLR	Toll-like receptor

TNF α	Tumour necrosis factor alpha
TNFR1	Tumour necrosis factor receptor 1
TSS	Transcription start sites
TST	Tuberculin skin test
UK	United Kingdom
μ l	Microlitre
USA	United States of America
UTR	Untranslated region
VDR	Vitamin D receptor
WBA	Whole blood assay
WHO	World Health Organisation
XCPEI	X core promoter element I

List of Amino Acid Codes

A	Ala	Alanine
R	Arg	Arginine
N	Asn	Asparagine
D	Asp	Aspartic acid
C	Cys	Cysteine
Q	Gln	Glutamine
E	Glu	Glutamic acid
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
L	Leu	Leucine
K	Lys	Lysine
M	Met	Methionine
F	Phe	Phenylalanine
P	Pro	Proline
S	Ser	Serine
T	Thr	Threonine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
V	Val	Valine
X		Stop codon

CHAPTER 1:

An overview of the IL12/IL23/IFN γ pathway and genetic susceptibility to mycobacterial infection and *Mycobacterium tuberculosis*

1.1 Introduction

Tuberculosis (TB) has caused the death of more people than any other single infectious agent, making it the most successful pathogen known to man. In Europe at the beginning of the nineteenth century the mortality was between 200 and 300 per 100 000 population. Today, the most optimistic global scenarios predict in excess of 80 million new cases and 20 million deaths in the next decade, 95% of which will occur in resource-poor countries.

TB remains an important cause of morbidity and has only been displaced from the position of leading infectious cause of death by the arrival of the human immunodeficiency virus (HIV).

Approximately one third of the human population is infected with *Mycobacterium tuberculosis* (*M. tb*), but the disease will only develop from latent infection to active disease in five - ten percent of immunocompetent individuals (WHO Report 2011: Global tuberculosis Control). Estimated global TB incidence rates per 100 000 population are shown in figure 1.1.

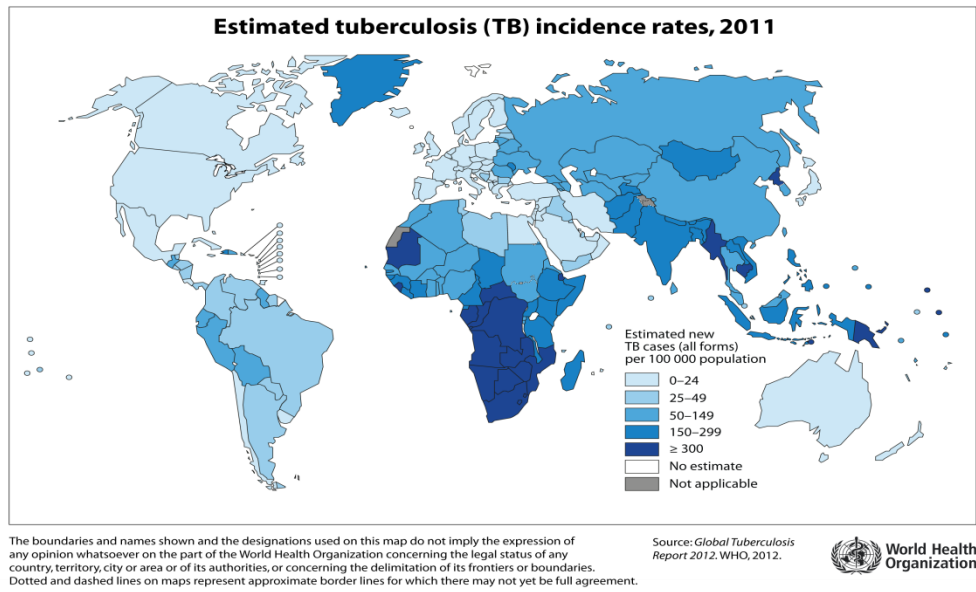


Figure 1.1: Estimated global TB incidence rates 2011.

Source: http://gamapserver.who.int/mapLibrary/Files/Maps/Global_TB_incidence_2011.png

There are many factors predisposing people to infection with mycobacterial species, including *M. tb*. These include environmental factors like malnutrition and poor socio economic living conditions, Mendelian disorders affecting immune function, such as Severe Combined Immunodeficiency (Marchand *et al.* 2008), X-linked Chronic Granulomatous Disease (CGD) (Bustamante *et al.* 2007a, Lee *et al.* 2008) and cystic fibrosis (Giron *et al.* 2008), secondary immunodeficiencies such as HIV (Bekker and Wood 2010) and autoimmune disease (Harris and Keane 2010).

Aside from HIV, the strong association between poverty and TB due to overcrowding, inadequate ventilation and malnutrition allow TB infection to be more readily transmitted. Many of the poorest countries of the world have experienced massive population expansion that, together with limited resources for infrastructure development

and poor civic planning, have led to sprawling informal settlements. It is estimated that about 80% of the urban population of these countries live in slums (UN-Habitat, 2013). The poor socio-economic and environmental conditions characterizing these areas, facilitate the transmission of infectious diseases, including TB.

Evidence suggests that there are genetic components responsible for a predisposition to mycobacterial infection.

1.2 Approaches used in Genetic Studies

Different approaches have been used for the identification of genes responsible for susceptibility or resistance to disease (Casanova and Abel 2002, Burgner *et al.* 2006).

The Human Genome Project (HGP) opened up avenues for understanding the molecular basis of health and disease (Peltonen *et al.* 2001). Prior to the HGP, understanding of the molecular basis of disease evolved through family based linkage studies based on clinical data and association studies, to map the genes responsible for Mendelian disorders (Glazier *et al.* 2002). Several methods can be used for each approach.

Studies of inbred families with two or more affected siblings look for genetic linkage by searching for areas of the genome which have alleles that are homozygous in all affected family members (Newport *et al.* 1996). Microsatellite markers can be used to scan an entire genome for alleles that are shared by only the affected family members. Once a locus has been identified, further studies can be carried out to find the genetic variant. This approach was often favoured as it avoided the need for a hypothesis about the candidate gene (Newport 2009). It is however unlikely to detect any loci that

exert a moderate effect on disease susceptibility, so is best suited for study of single gene disorders.

Affected sibling pair (ASP) methodology, which looks for genetic similarities in siblings who share a trait was often used, because, unlike other linkage approaches, the mode of inheritance (*eg* recessive or dominant) was not required to be known. The ASP approach also avoids bias that could be introduced if healthy subjects were classified as resistant to disease when they were genetically susceptible, but not exposed to environmental trigger. The main limitation of ASP studies is the lack of power for detecting genes with weak effects, likely to be the case with complex diseases like insulin-dependent diabetes (Davies *et al.* 1994, Kapur *et al.* 2012).

Previously, association studies, searching for genetic differences between unrelated cases and controls in populations as opposed to families, had more power, but were limited to candidate genes, due to the large number of polymorphisms that would need to be typed for a genome- wide approach (Freimer *et al.* 2004). Genome wide association studies have extended the scope of genetic studies and today are widely used in genetic susceptibility studies.

Case control studies are often used in candidate gene association studies. Once a candidate gene has been identified, the whole gene may be sequenced in a small cohort, looking for single nucleotide polymorphisms (SNPs). The frequency of the variant allele is measured in cases and controls, looking for association. As SNP frequencies can differ between populations, resulting in conflicting results, very large numbers of cases and controls are required for such studies to be valid.

As the HGP, progressed, parallel projects paved the way for genome wide association studies (GWAS) for multifactorial diseases. These were the Single Nucleotide Polymorphism Consortium that reported 1.42 million SNPs distributed throughout the human genome (The international SNP Map Working Group 2001), the International HapMap project and a second generation human haplotype map of over 3.1 million SNPs from genotyping 270 individuals from four geographical diverse populations (The International HapMap Consortium 2003, International HapMap Consortium 2007).

Genome wide association studies (GWAS) central provides a centralized database of genetic association studies, collating findings from studies and listing publications (Newport and Finan 2011, www.gwascentral.org). In this way, GWAS are able to identify common genetic factors that influence disease that could be targets for new vaccines and drugs. The Wellcome Trust Case Control Consortium (WTCCC) conducted a GWAS study of 14 000 cases of seven common disorders and 3000 shared controls, demonstrating the feasibility of large scale studies (Wellcome Trust Case Control Consortium 2007).

Animal studies have evaluated genetic susceptibility to infectious diseases, including tuberculosis. A study done by Bekker *et al.* 2000, using a Tumour Necrosis Factor Alpha (TNF α) knockout murine model looked at immunopathologic effects of mycobacterial infection. Tobin *et al.* 2010 carried out a genetic screen on zebrafish larvae and identified mutant classes showing susceptibility to *Mycobacterium marinum*. A hypersusceptible mutant mapped to *Ita4h* locus, encoding leukotriene A₄ hydrolase, which catalyses the final step in the synthesis of leukotriene B₄, a chemoattractant and proinflammatory eicosanoid. The resultant anti-inflammatory state was shown to

permit mycobacterial infiltration by limiting production of $\text{TNF}\alpha$, a key anti-inflammatory molecule. This research group extended the animal work to humans, observing that significant protection from tuberculosis and leprosy were associated with heterozygosity for LTA4H polymorphisms in Vietnamese and Nepali cohorts respectively. Conversely, Curtis *et al.* 2010 genotyped six LT4AH gene polymorphisms in samples from 9115 Russian subjects (3703 patients with pulmonary tuberculosis and 5412 healthy controls) and found no evidence that these common polymorphisms provide protection against pulmonary tuberculosis.

Today, many genes are being identified through microarray gene chip and gene expression technologies, allowing quicker and more efficient identification of candidate genes (Mistry *et al.* 2007, Berry *et al.* 2010, Lesho *et al.* 2011, Maertzdorf *et al.* 2012, Verhagen *et al.* 2012, Anderson *et al.* 2014). Blood transcriptional profiling has improved diagnosis and understanding of TB.

The different approaches used for genetic studies are summarized below in table 1.1.

Approach	Requirements	Advantages	Disadvantages
Family studies			
Genetic linkage	2 or more affected siblings and parents	Single gene disorders detected	Loci with moderate effect not detected
ASP	Affected siblings	Mode of inheritance not required Bias avoided	Lack of power
Association studies			
Case control studies	2 or more affected siblings and matched controls	More power	Limited to candidate genes
GWAS studies	Large numbers of cases and controls	Good for studying complex diseases Large scale screening	Rare variants not identified
Gene expression studies			
Microarray, gene chip, gene expression	Moderately large numbers of cases and controls	Large scale screening, fast, no limitation for detection of variants	Cost
Genome capture studies			
Exome capture	Large numbers of cases and controls	More power, unbiased investigation of complete coding region	Cost, may miss mutations in regulatory and non-coding regions

Table 1.1: Approaches used in genetic studies

1.3 Early association studies and identification of the Mendelian susceptibility genes of the IL12/IL23/IFN γ pathway

Using technology available in the 1990s, the main candidate genes that appeared to be associated with mycobacterial disease were investigated by linkage analysis and association studies. These studies

looked at significant associations between specific gene polymorphisms and phenotypes within families and populations.

This led to the identification of Mendelian susceptibility genes of the IL12/IL23/IFN γ pathway that may be mutated in children and adults with a predisposition to mycobacterial disease.

The IL12/IL23/IFN γ pathway, also known as the type I cytokine pathway, plays an important role in the protection of individuals against mycobacterial infection. This pathway consists of several genes including interferon gamma receptor 1 (IFN γ R1), interferon gamma receptor 2 (IFN γ R2), interferon gamma (IFN γ), interleukin 12 receptor beta 1 (IL12R β 1), (interleukin 12 receptor 2 (IL12R β 2), interleukin 12 (IL12) and signal transducer and activator of transcription 1 (STAT1) (Casanova 2001, Doffinger *et al.* 2001). Molecular analysis of patients with defects in the genes of this pathway has provided evidence of the genetic component and highlighted the importance of the IL12/IFN γ pathway in host defence against intracellular pathogens, including mycobacteria, Salmonella and some viruses (Casanova 2001). About a third of patients with idiopathic disseminated poorly pathogenic mycobacteria and/or systemic salmonella infections have been found to have mutations in the genes of this pathway (Altare *et al.* 1998d). Parental consanguinity and familial forms are frequently observed in affected individuals and this syndrome has subsequently been designated Mendelian Susceptibility to Mycobacterial Disease (MSMD).

Patients with MSMD caused by single gene defects in the IL12/IL23/IFN γ pathway are primarily susceptible to non-tuberculous mycobacterial infections (NTM), but may also develop TB. The study

of patients with NTM infections has added to our understanding of the mechanisms responsible for susceptibility to disease from *M. tb*.

The following is a description of the major genes of the IL12/IL23/IFN γ pathway.

1.3.1 Major genes of the IL12/IL23/IFN γ pathway associated with MSMD

1.3.1.1 Interferon gamma receptor 1 and 2 (IFN γ R1 and R2)

The gene loci for IFN γ R1 and IFN γ R2 are chromosomes 6q23-6q24 and 21q22.1-22.2 and each gene has seven exons (Soh *et al.* 1994, Farrar and Schreiber 1993).

Functional IFN γ receptors are composed of two 90 kDa IFN γ R1 (ligand binding chain) proteins and two 62 kDa IFN γ R2 (signal transducing chain) proteins. Both IFN γ R1 and IFN γ R2 are expressed by phagocytic and dendritic cells (Pfizenmaier *et al.* 1988, Soh *et al.* 1994, Cook *et al.* 1994).

The extracellular portion of IFN γ R1 contains the ligand binding domain and the intracellular portion contains domains necessary for signal transduction and receptor recycling (Farrar *et al.* 1991, Kotenko *et al.* 1995, Bach *et al.* 1997).

IFN γ signaling is an ordered, affinity driven process that derives its specificity from the specific binding site of a particular signal transducer and activator of transcription (STAT) protein to a defined, ligand-induced docking site on the activated receptor and the ability of the STAT1 homodimer to specifically activate IFN γ induced gene transcription (Bach *et al.* 1997).

Two IFN γ proteins bind as a homodimer to the two IFN γ R1 ligand binding chains that then dimerise and become associated with two IFN γ R2 signal transducing chains.

The extracellular domain of IFN γ R2 interacts with the IFN γ R1/IFN γ complex (Bach *et al.* 1997).

Receptor assembly leads to activation of the JAK1 and JAK2 and phosphorylation of a tyrosine residue on the intracellular domain of IFN γ R1. The schematic sequence of these events is depicted in figure 1.2.

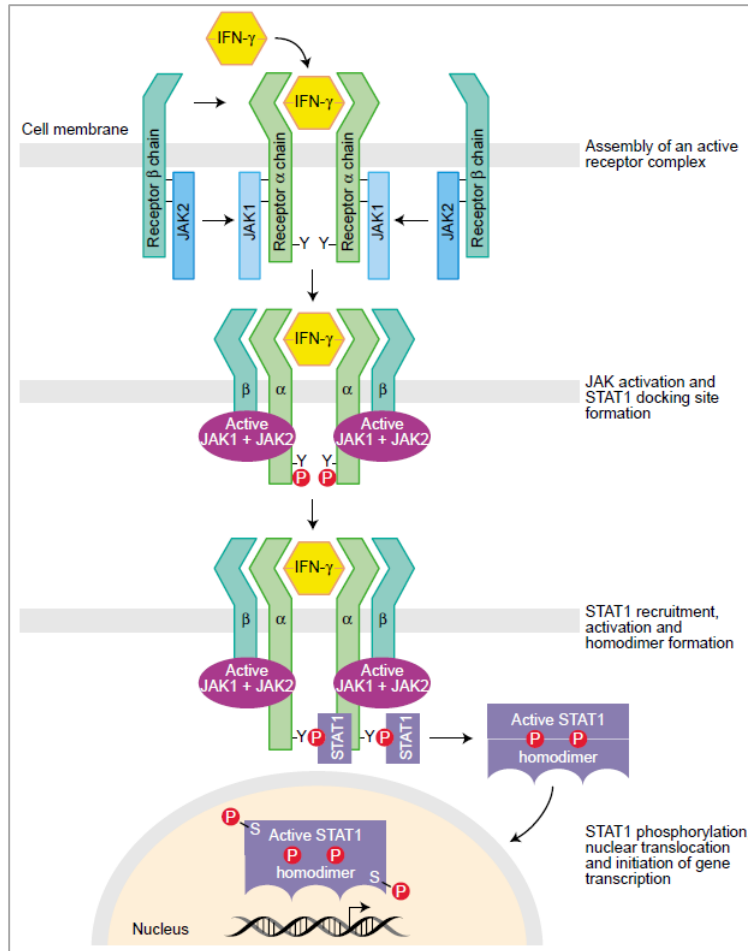


Figure 1.2: Schematic representation of the IFN γ signaling pathway.

Source: Newport 2003, modified figure reproduced from Bach *et al.* 1997

Within the IFN γ R1/IFN γ complex, the IFN γ R1 and IFN γ R2 intracellular domains with their constitutively associated JAKs are brought into proximity. Ligand binding results in reciprocal transphosphorylation of IFN γ R1 tyrosine 440 (Y440) (Bach *et al.* 1997, Igarashi *et al.* 1994).

Through its SH2 domain, one latent STAT1 recognizes and binds to each tyrosine phosphorylated IFN γ R1 440tyrosine-aspartic acid-lysine-proline-histidine444 (440YDKPH444) site. The amino acid binding sites are depicted in figure 1.3.

Receptor associated STAT1 proteins are subsequently tyrosine phosphorylated, activated and form homodimers. They are then phosphorylated and released into the cytosol as phosphorylated STAT1 homodimers, forming gamma activating factors (GAFs) which are translocated to the nucleus. GAFs bind gamma activating sequences (GASs) present in the promoters of target genes (Chen *et al.* 1998). After signaling, the ligand binding chains are internalized, dissociate and recycled to the cell surface (Shuai *et al.* 1992, Shuai *et al.* 1993).

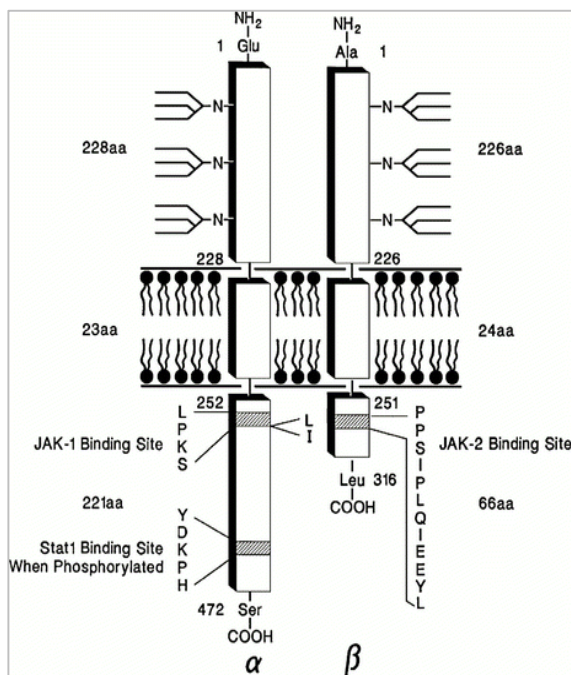


Figure 1.3: IFN γ signaling showing amino acids of binding sites.

Source: Bach *et al.* 1997

1.3.1.2 Interferon gamma (IFN γ)

Interferon family members have been segregated into two categories. Type I IFN is induced by cells as a result of viral infection and has been divided into two classes, based on cell origin. IFN α is a family of 17 related proteins encoded by specific genes that are synthesized mainly

by leukocytes. IFN β is a single protein encoded by a specific gene produced mainly by fibroblasts.

Type II IFN, commonly known as IFN γ is induced by immune and inflammatory stimuli and is synthesized predominantly by T lymphocytes and NK cells (Bach *et al.* 1997).

The human IFN γ molecule is a non covalent homodimer consisting of two identical 17kD polypeptide chains (Gray *et al.* 1982) and was first identified on the basis of its *in vitro* antiviral activity (Wheelock 1965). The gene locus for IFN γ is chromosome 12q24. There are four exons and a non-specific enhancer element in the first intron.

IFN γ activates the transcription of many genes that play roles in antiviral activity, apoptosis, MHC protein expression, antigen processing and type I T helper cell (Th1) development. IFN γ also activates macrophages to kill or restrict growth of microbial targets. This function appears to be important in host defense against mycobacteria. In an inflammatory reaction, IFN γ is induced by IL12 and IL18 secreted by macrophages and dendritic cells (Kobayashi *et al.* 1989, Seder *et al.* 1993, Micalief *et al.* 1996, Song *et al.* 2002).

IFN γ stimulates the development and function of immune effector cells and also regulates the production of various other immunomodulatory or proinflammatory cytokines such as IL12 and TNF α (Bach *et al.* 1997). As IFN γ plays such a major role in stimulating antimicrobial functions of monocytes and macrophages, people with defective receptors acquire disseminated infections with mycobacterial species of low virulence (Holland 2000).

1.3.1.3 Interleukin 12 receptor β 1 and β 2 (IL12R β 1 and β 2)

The IL12R is composed of two chains, IL12R β 1 and IL12R β 2, both of which have extensive homology to gp130, the common receptor β chain of the IL6-like cytokine superfamily. IL12R and IL23R share the same IL12R β 1 subunit.

The gene locus for IL12R β 1 is chromosome 19p13.1 and there are 16 exons. The gene locus for IL12R β 2 is chromosome 1p31.2 and there are 15 exons (Yamamoto *et al.* 1997).

The two receptor proteins are type 1 transmembrane glycoproteins. Functional IL12 receptors are expressed primarily on activated T and NK cells (Desai *et al.* 1992) and co-expression of the two receptor units is required for high affinity binding of IL12. The IL12R β 2 subunit functions as the signal transducing component of the high affinity receptor complex (Presky *et al.* 1996), can be expressed at the cell surface in the absence of IL12R β 1 and can be upregulated by IFN γ (Verhagen *et al.* 2000).

Signal transduction through IL12R induces tyrosine phosphorylation, primarily of JAK2 and TYK2, which in turn phosphorylate and activate STAT1, STAT3, STAT4 and STAT5. The specific cellular effects of IL12 are due mainly to its ability to induce activation of STAT4. Phosphorylated STAT4 molecules then dimerise and translocate to the nucleus, where IL12 inducible genes with an interleukin (IL) response element (IL12RE) are activated (Holland 2010). The IL12 signaling is depicted in figure 1.4.

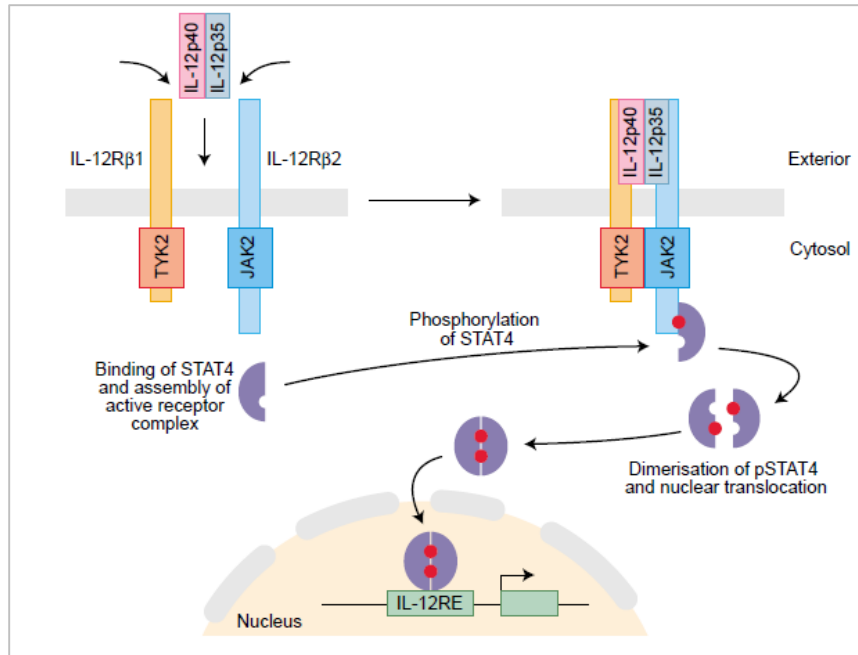


Figure 1.4: schematic representation of the IL12 signaling pathway.

Source: Newport 2003, modified figure reproduced from Lammas *et al.* 2000

1.3.1.4 Interleukin 12 (IL12)

IL12 is a heterodimer of 70 000 daltons (IL12p70) comprised of two covalently linked glycosylated subunits of 40 000 (IL12p40) and 35 000 (IL12p35) daltons respectively (Kobayashi *et al.* 1989).

The gene locus for heavy chain IL12p40, also known as IL12B is 5q31.1-q33.1. The gene has 7 exons, the first of which is untranslated. The gene locus for light chain IL12p35, also known as IL12A is 3p12-q13.2 and this gene also has 7 exons.

In human peripheral blood, IL12 is mainly produced by phagocytes (monocytes/macrophages and neutrophils) and dendritic cells in response to pathogens (bacteria, fungi, intracellular parasites and viruses) as a result of soluble signals from activated T cells, NK cells and components of the inflammatory extracellular matrix (Cassatella

et al. 1995, D'Andrea *et al.* 1992, Trinchieri 2003). The biology of these components is depicted in figure 1.5.

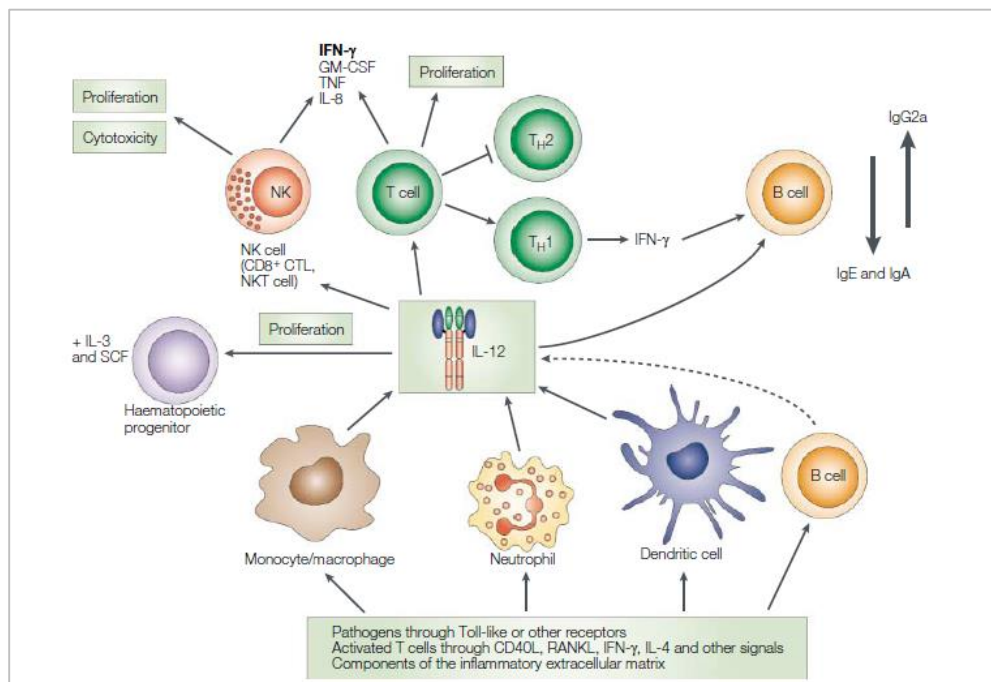


Figure 1.5: Schematic representation of the biology of IL12.

Source: Trinchieri 2003

IL12 plays an important role in promoting Th1 responses and subsequent cell mediated immunity (Hsieh *et al.* 1993, Manetti *et al.* 1993, Afonso *et al.* 1994). Production of IL12 stimulated by microbial lipoproteins, including a 19 kD *M. tb* lipoprotein is mediated by TLRs (Brightbill *et al.* 1999).

IL12 enhances proliferation and cytotoxic activity of NK cells and T cells and stimulates their IFN γ production which together with other secondary and tertiary pro-inflammatory cytokines, have a direct toxic effect on tumour cells (Trinchieri 1994).

In addition to the augmentation of cellular immune responses, IL12 also augments the production of opsonizing and complement-fixing

classes of IgG antibodies that have been shown to have anti-tumour activity *in vivo* (Quaglino *et al.* 2002).

IL12p40 associates not only with IL12p35 to form IL12p70, but also with p19 to form a heterodimeric cytokine IL23 (Oppmann *et al.* 2000), shown in figure 1.6 Although IL23 and IL12p40 share the common p40 subunit, these cytokines have divergent activities (Oppmann *et al.* 2000, Langrish *et al.* 2005).

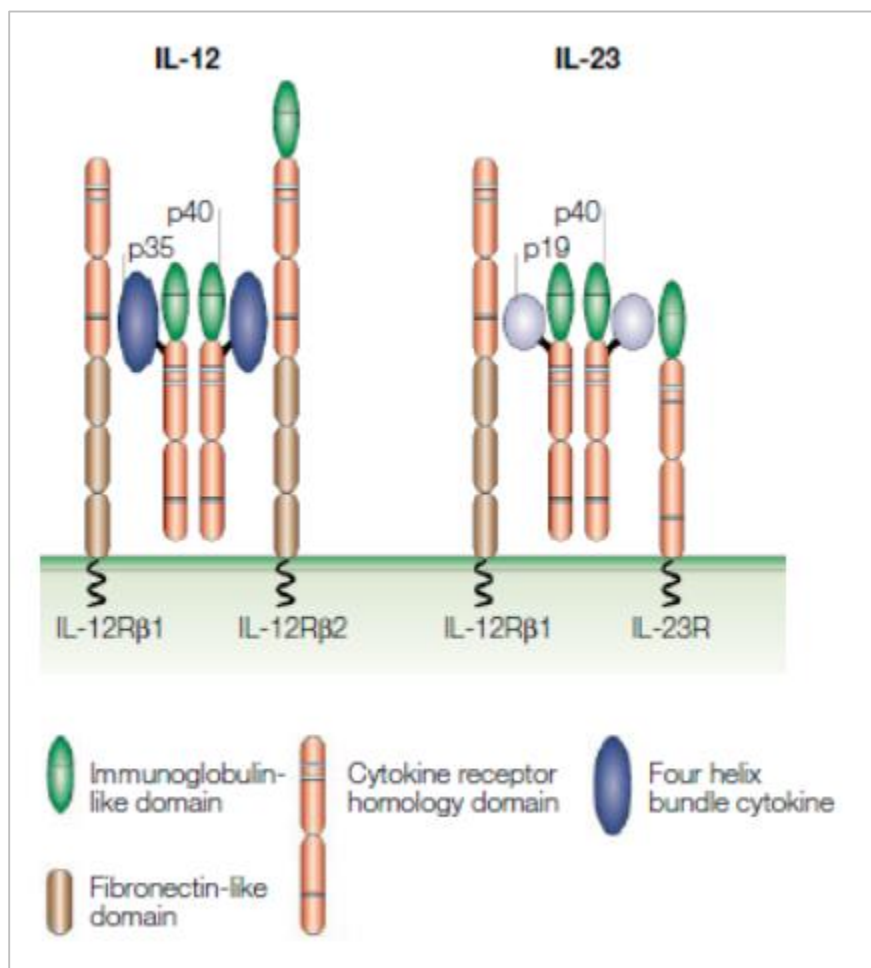


Figure 1.6: The subunits of IL12 and IL23.

Source: Trinchieri 2003

Activated dendritic cells secrete detectable levels of IL23 which bind to IL12R β 1 and a second chain IL23R (Parham *et al.* 2002).

IL23 induces the same JAK-STAT signaling molecules (JAK2, TYK2, STAT1, STAT3, STAT4 and STAT5) as does IL12, but it induces different DNA binding STAT dimers (Parham *et al.* 2002), possibly explaining the overlapping but not identical activities of the two cytokines. Similar to IL12, human IL23 stimulates IFN γ production and proliferation in PHA blast T cells as well as in CD45RO memory T cells (Oppmann *et al.* 2000). IL23 is an essential factor required for the expansion of a pathogenic CD4⁺ T cell population, which is characterized by the production of IL17, IL17F, IL6 and TNF.

Humans lacking the common IL12p40 subunit are particularly susceptible to mycobacterial disease (Filipe-Santos *et al.* 2006) and this deficiency may reflect a role of both IL12 and IL23 in control of infection.

IL12p40 and IL12R β 1 deficiencies also cause defects in IL23 and IL23R respectively. As IL12 and IL23 share the p40 subunit, IL12p40 deficiency should cause both defective IL12 and IL23 function. Likewise, as IL12R and IL23R use the IL12R β 1 subunit, IL12R β 1 deficiency should cause defective IL12R and IL23R function (van de Vosse *et al.* 2004).

Pedraza *et al.* 2010 reported two IL12R β 1 deficiency patients affected with *Klebsiella pneumoniae* infections. They hypothesized that the infections could be due to a limited capacity to produce IL17. This impaired IL23-dependent IL17 immunity in IL12p40 and IL12R β 1 deficiency patients may explain why Salmonella infection is 10X more

frequent in these patients than in IFN γ R deficiency patients whose IL23/IL17 circuit is intact.

In contrast to the protective role for IL23, there have also been reports of increased pathologic consequences related to IL23 (Cruz 2010). This highlights the fact that for chronic disease like TB, a balance in the level of IL23 is important. High levels of the cytokine may be detrimental and with low levels, there is loss of protection against disease (Khader *et al.* 2011).

1.3.1.5 Signal transducers and activators of transcription (STAT)s

STATs serve as links between the cell surface and the nucleus and this requires their physical movement from the cytoplasm to the nucleus.

STATs are DNA binding factors that are regulated directly by tyrosine phosphorylation (Shuai *et al.* 1993), which confers new properties to STAT factors by inducing dimerization via reciprocal phosphorylation and Src homology 2 (SH2) domain interactions. This conformational change is essential for binding to specific DNA targets.

The gene locus for STAT1 is 2q32.2, there are 23 exons, the first of which is untranslated. STAT1 was originally identified as a latent cytosolic transcription factor involved in signaling of IFN α for antiviral immunity and IFN γ for anti-mycobacterial immunity and has since been shown to be activated by a wide variety of cytokines and growth factors (Darnell *et al.* 1994). In response to cytokines and growth factors, STAT1 family members are phosphorylated by the receptor kinases. They then form homodimers or heterodimers that translocate to the cell nucleus, where they act as transcription factors. The STAT proteins can be activated by various ligands, including IFN γ .

STAT1 signaling is shown in figure 1.7.

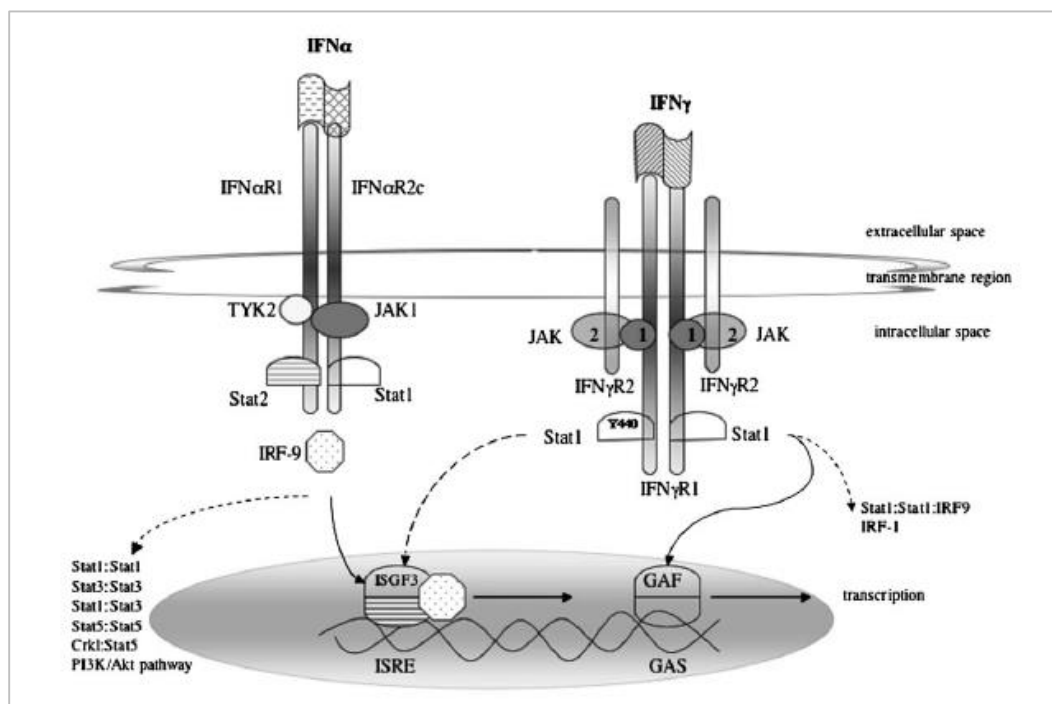


Figure 1.7: IFN γ and IFN α signaling showing antiviral immunity by IFN α and anti-mycobacterial immunity by IFN γ .

Source: Haverkamp *et al.* 2006

STAT4 has also been shown to be important for protection against mycobacterial disease. A study done by Sabri *et al.* 2014 showed association between a STAT4 SNPs and pulmonary TB in a Moroccan population.

STAT4 was initially cloned as a result of its homology with STAT1 (Yamamoto *et al.* 1994), is widely expressed and is only tyrosine phosphorylated after stimulation of T cells with IL12. Experiments with mice lacking STAT4 showed that all IL12 functions were disrupted, including the induction of IFN γ (Wang *et al.* 2014).

Kim *et al.* 2008 reported decreased phosphorylation of STAT1 and STAT4 in patients with multi drug resistant TB.

Toyoda *et al.* 2004 have examined the immunological abnormality in a patient with recurrent *Mycobacterium avium* (*M. avium*) infection and the defect seemed likely to be impaired STAT4 nuclear translocation in IL12 stimulated T cells, leading to impairment of IFN γ production.

1.3.1.6 IL12/IL23/IFN γ pathway interactions

When macrophages are infected with mycobacteria, biologically active IL12p70 binds to the high affinity receptor IL12R on NK cells, CD4+T helper cells and cytotoxic T cells. Binding of IL12 to the heterodimeric IL12R complex induces intracellular events, resulting in the activation of IL12 inducible genes including those encoding IFN γ . The secretion of IFN γ by NK and T cells elicits a cell mediated immune response to intracellular pathogens within the infected macrophage by binding to the IFN γ Rs on the macrophages. As the ligand binding chains interact with the IFN γ , they dimerise and induce intracellular events that activate, via STAT1, a range of IFN γ responsive genes. This sequence of events is depicted in figure 1.8.

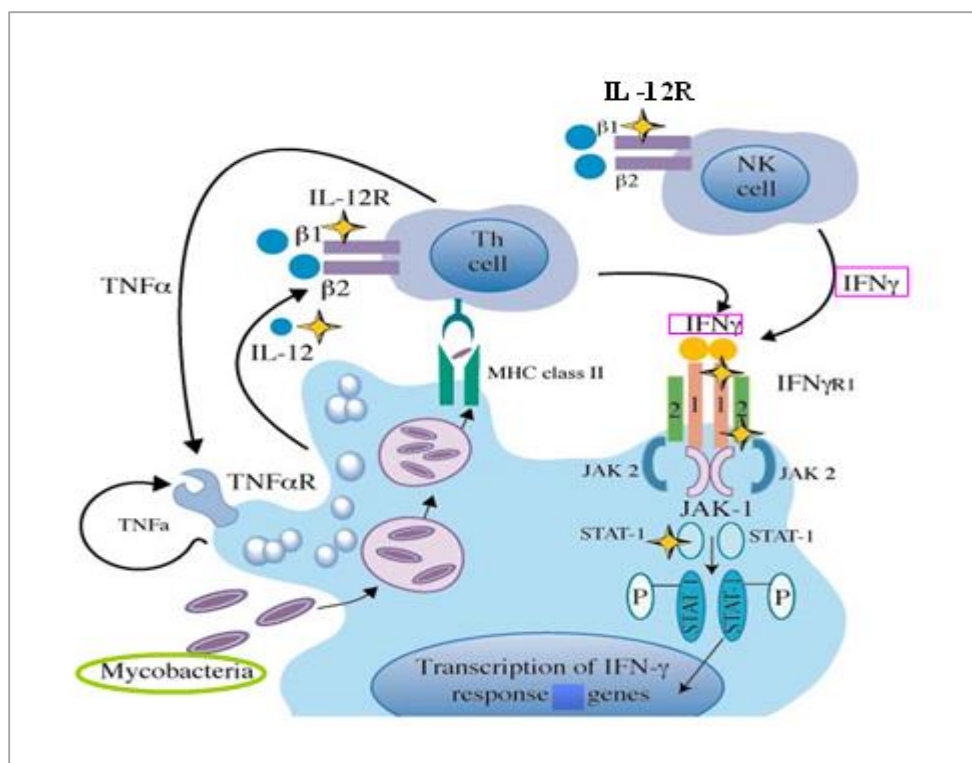


Figure 1.8: Overview of the IL12/IL23/IFN γ pathway, including signal transduction and transcription of IFN γ response genes, with stars denoting proteins that are encoded by mutated genes associated with MSMD.

Source: Dr S. Anderson 2002 (personal communication)

1.4 Reported Mutations of the IL12/IL23/IFN γ Pathway Causing MSMD

The elucidation of the critical role of IFN γ R genes in control of mycobacterial infection came about with the identification of kindreds in whom affected individuals had severe infection with poorly virulent NTM in the absence of a known immunodeficiency (Uchiyama *et al.* 1981, Newport and Levin 1994).

IFN γ R1 deficiency was the first human mycobacterial susceptibility gene to be identified (Newport *et al.* 1996, Jouanguy *et al.* 1996).

Four children from the same small town in Malta where intermarriage between families is common, presented with disseminated atypical mycobacterial infection in the absence of any recognized immunodeficiency. Each child was infected with a different species or strain of mycobacterium, viz *M. fortuitum*, *M. avium* and *M. chelonii*, as well as severe recurrent infections with *Salmonella*, suggesting a defect in host immunity.

The partial consanguinity suggested a Mendelian disorder of autosomal recessive inheritance (Levin and Newport 1995).

Immunologic investigation showed reduced $\text{TNF}\alpha$ production in response to stimulation with $\text{IFN}\gamma$ plus endotoxin in a whole blood assay (Levin *et al.* 1995). A subsequent genome-wide search using microsatellite analysis identified a region on chromosome 6q for which all affected children in this family were homozygous (Newport *et al.* 1996). The gene encoding $\text{IFN}\gamma\text{R1}$ was known to map to that chromosomal region and this was further investigated. Lymphocytes from patients were shown to lack expression of $\text{IFN}\gamma\text{R1}$ protein and DNA sequencing revealed that the affected patients were homozygous for a point mutation C395A that resulted in a premature termination codon S116X.

At the same time, the report of a Tunisian infant with disseminated vaccine associated BCG infection and a different mutation 131delC in the $\text{IFN}\gamma\text{R1}$ gene, also resulting in premature termination TAA at 187-189 (Jouanguy *et al.* 1996) firmly established the importance of $\text{IFN}\gamma$ responsiveness in control of both vaccine associated BCG infection and environmentally acquired NTM infections.

Various IFN γ R mutations have since been described in individuals from many countries and in many ethnic groups.

These patients with single gene defects in the IFN γ signaling pathway are rare, but genetic, immunological and clinical study of the consequences of the defects has led to invaluable insight into human control of mycobacterial infection. The molecular analyses of these first kindreds, assisted in the understanding of these inherited immunodeficiencies.

Genetic defects continue to be reported on an ongoing basis. The following is a description of some of the early ones identified that assisted in the understanding of these inherited immunodeficiencies.

1.4.1 IFN γ R Deficiencies

1.4.1.1 Complete IFN γ R1 Deficiency

Reported mutations of complete IFN γ R1 deficiency are all autosomal recessive and two forms have been reported.

In one form, the patients have been shown to harbour null mutations that prevent receptors from being expressed as in the first IFN γ R1 mutations described, C395A/S116X (Newport *et al.* 1996) and 131delC (Jouanguy *et al.* 1996).

In the other form of complete IFN γ R1 deficiency, patients were found to have normal cell surface expression of the receptor, but mutations in the extracellular ligand binding domain, prevented the surface receptors from binding the IFN γ . The first mutations of this kind, 295del12 and G230C/C77Y were reported by Jouanguy *et al.* 2000.

Patients with complete IFN γ R deficiencies have high plasma IFN γ levels (Fieschi *et al.* 2001).

1.4.1.2 Partial IFN γ R1 Deficiencies

Partial IFN γ R1 deficiency presents with a milder phenotype and mutations can be autosomal recessive (ARIFN γ R1), resulting in later onset of infection or the dominant negative (ADIFN γ R1) form.

Partial recessive IFN γ R1 deficiency was first reported, presenting with a clinically heterogenous disease (Jouanguy *et al.* 1997b, Remiszewski *et al.* 2006).

A partial dominant form of IFN γ R1 deficiency was first identified independently in 12 unrelated kindreds, associated with a mutation hotspot in the IFN γ R1 gene (Jouanguy *et al.* 1999).

These patients were found to have heterozygous frame shift small deletions in exon 6 of the IFN γ R1 gene. The mutant alleles encode truncated receptors that bind IFN γ with normal affinity, but accumulate at the cell surface due to the lack of an intracellular recycling site, resulting in hyperexpression.

The small deletions of 4 nucleotides, either AATT, ATTA, TTAA or TAAT at nucleotides 816, 817, 818 or 819 found in eleven families, were arbitrarily designated 818del4 and the 1 nucleotide deletion 818delT or 819delT, found in one family was arbitrarily designated 818delT (Jouanguy *et al.* 1999)

Nucleotide 818 of the IFN γ R1 gene was the first small deletion hotspot to be identified in the human genome (Doffinger *et al.* 2000b) and these overlapping small deletions at a single mutational site have been globally designated as 818 deletions.

A mutation hotspot is defined as a site at which at least two independent mutational events of the same type occur. Small deletion mutational hotspots may have direct nucleotide repeats in flanking areas, which are thought to increase the risk of slipped mispairing during DNA replication. Polypurine runs have also been shown to be associated with small deletions (Krawczak *et al.* 1991). This model of slipped mispairing events and subsequent repair during replication has been proposed, based on the presence of two direct repeats in the vicinity of nucleotide 818.

The IFN γ R1 818del4 is the most common mutation occurring in this gene and since this early report, it has been found in other families from many countries.

Another hotspot in the IFN γ R1 gene was reported in an Argentinian patient by Rosenzweig *et al.* in 2002. Due to the nucleotide sequences flanking the region, the deletion could be ACTC, CTCA or TCAC at nucleotides 561, 562 or 563, resulting in the same sequence ATACGC and was arbitrarily designated 561del4. This 561del4 mutation occurs in the vicinity of direct AC repeats and a polypurine run.

The 561del4 mutation had been previously identified in a German patient with a compound heterozygous mutation which included the maternally inherited 561del4 and a paternally inherited splice site mutation 373+1G \rightarrow T (Roesler *et al.* 1999). Unlike the 818del4 which is dominantly inherited, the 561del4 mutation is recessive in both the Argentinian and German cases.

Additional detailed IFN γ R1 gene mutations are listed in table 1.2.

Mutation	Phenotype	Effect of mutation	Country of origin	Infections	Reference
C395A/S116X (4 children)	Complete recessive	Premature stop codon, no detectable protein expression	Malta	<i>M. fortuitum</i> , <i>M. avium</i> , <i>M. chelonii</i> +	Newport <i>et al.</i> 1996
131delC	Complete recessive	Premature stop codon, no detectable protein expression	Tunisia	Disseminated BCG	Jouanguy <i>et al.</i> 1996
200+1G→A splice site and 107insA, compound heterozygote	Complete recessive	Skipping of exon 2, premature stop codon, no detectable protein expression	Italy	<i>M. smegmatis</i>	Reported by Pierre-Audigier <i>et al.</i> 1997, later described by Altare <i>et al.</i> 1998b, Dorman <i>et al.</i> 2004
T260C/I87T	Partial recessive	Expression of nonfunctional protein	Portugal, Poland	Disseminated BCG, <i>M. pneumonia</i> , <i>M. avium</i>	Jouanguy <i>et al.</i> 1997b, Remiszewski <i>et al.</i> 2006, van de Wetering <i>et al.</i> 2010
22delC	Complete recessive	Premature stop codon, no detectable protein expression	Pakistan (born in Norway)	<i>M. avium</i>	Holland <i>et al.</i> 1998
201-2A→G splice site (2 families)	Complete recessive	Deletion of exon 3, no protein expression	India, Pakistan	<i>M. avium</i>	Holland <i>et al.</i> 1998, Dorman <i>et al.</i> 2004
Not described	Complete recessive	No detectable protein expression	Pakistan (living in Norway)	Disseminated BCG, <i>M. avium</i>	Vesterhus <i>et al.</i> 1998
373+1G→T splice site and 561del4, compound heterozygote	Complete recessive	Deletion of exon 3 and small deletion in exon 5, no detectable protein expression	Germany	<i>Listeria monocytogenes</i> , disseminated BCG, <i>M. kansasii</i> , <i>M. avium/intracellulare</i>	Roesler <i>et al.</i> 1999
818del4 (many families)	Partial dominant	Overexpression of nonfunctional protein	UK, Germany, Italy, Morocco, Sweden, USA, India, France, Netherlands, Indonesia, Japan	Disseminated BCG, <i>M. avium</i> , BCG osteomyelitis, <i>M. kansasii</i> , <i>Histoplasma capsulatum</i>	Razka <i>et al.</i> 1994, Jouanguy <i>et al.</i> 1999, Villela <i>et al.</i> 2001, Arend <i>et al.</i> 2001, Waibel <i>et al.</i> 2002, Han <i>et al.</i> 2004, Zerbe <i>et al.</i> 2005, Sasaki <i>et al.</i> 2002, de Boer <i>et al.</i> 2008, Lee <i>et al.</i> 2009
818delT	Partial dominant	Overexpression of nonfunctional	Italy	<i>M. avium</i> osteomyelitis.	Jouanguy <i>et al.</i> 1999

		protein		disseminated BCG	
295del12	Complete	Normal expression of nonfunctional protein	Algeria	Disseminated BCG, <i>M. avium</i>	Jouanguy <i>et al.</i> 2000
G230C/C77Y	Complete recessive	Normal expression of nonfunctional protein	Turkey	HHV8 associated Kaposi sarcoma, <i>M. fortuitum</i> , disseminated BCG	Jouanguy <i>et al.</i> 2000, Camcioglu <i>et al.</i> 2004, van de Wetering <i>et al.</i> 2010
T182A/V61Q and deletion 652-654 or 653-655 designated 652del3 compound heterozygote	Complete recessive	Normal expression of nonfunctional protein	France/Portugal	Disseminated BCG	Jouanguy <i>et al.</i> 2000
165delG	Complete recessive	Premature stop codon, no detectable protein expression	Canada	Disseminated BCG	Cunningham <i>et al.</i> 2000
T832G/E278X	Partial dominant	Premature stop codon, no intracytoplasmic tail, overexpression of nonfunctional protein	USA	<i>M. avium</i> osteomyelitis	Villela <i>et al.</i> 2001, Dorman <i>et al.</i> 2004
T188G/V63G	Complete recessive	Normal expression of nonfunctional protein	Spain	<i>M. avium</i> , <i>M. szulgai</i>	Allende <i>et al.</i> 2001
811del4	Partial dominant	Overexpression of nonfunctional protein	Japan	BCG osteomyelitis	Sakai <i>et al.</i> 2002
523delT	Complete recessive	Premature stop codon	Italy	<i>M. peregrinum</i> , <i>M. scrofulaceum</i> bone lesions	Koscielniak <i>et al.</i> 2003, Marazzi <i>et al.</i> 2010
Intronic (CA) _n microsatellite marker	N/A	One allele protective, two alleles associated with disease (Fraser <i>et al.</i> 2003). No association found (Newport <i>et al.</i> 2003)	Croatia The Gambia	<i>M. tuberculosis</i>	Fraser <i>et al.</i> 2003, Newport <i>et al.</i> 2003
105insT	Complete recessive	Not reported	Turkey	<i>M. avium</i>	Dorman <i>et al.</i> 2004
168delC	Complete recessive	Not reported	USA	<i>M. avium</i>	Dorman <i>et al.</i> 2004
106_107insT/G197A	Complete recessive	Not reported	Greece	Mycobacterial species	Dorman <i>et al.</i> 2004
453delT	Complete	No detectable	Greece	<i>M. fortuitum-M.</i>	Tsolia <i>et al.</i> 2005

	recessive	protein expression		<i>peregrinum</i> complex	
C77F	Complete recessive	No detectable protein expression	Not reported	<i>M. fortuitum</i>	Chantrain <i>et al.</i> 2006
817insA	Partial dominant	Premature stop codon, overexpression of nonfunctional protein	Not reported	Not reported	Haverkamp <i>et al.</i> 2006
G254A/C71Y	Complete recessive	No detectable protein expression	Pakistan	Disseminated BCG, <i>M. avium</i>	Noordzij <i>et al.</i> 2007
373+1G→T, splice site, previously found in compound heterozygote	Complete recessive	Deletion of exon 3, absence of transmembrane domain, no detectable protein expression	Netherlands	<i>M. gordonae</i> , <i>M. peregrinum</i> , <i>M. mageritense</i> , <i>M. szulgai</i>	Noordzij <i>et al.</i> 2007
C85Y	Complete recessive	Minimal protein expression	Not reported	Not reported	van de Wetering <i>et al.</i> 2010
V61E, previously reported as V61Q by Jouanguy <i>et al.</i> 2000	Complete recessive	No detectable protein expression	Not reported	Not reported	van de Wetering <i>et al.</i> 2010
V61Q previously found in compound heterozygote	Complete recessive	Expression of nonfunctional protein	Not reported	Not reported	van de Wetering <i>et al.</i> 2010
V63G	Partial recessive	Severely reduced protein expression	Not reported	Not reported	van de Wetering <i>et al.</i> 2010
Y66C	Complete recessive	Cell surface expression of nonfunctional protein	Not reported	Not reported	van de Wetering <i>et al.</i> 2010

Table 1.2: Reported IFN γ R1 Deficiencies

1.4.1.3 Complete IFN γ R2 Deficiency

In 1998, Dorman and Holland reported the first child with complete IFN γ R2 deficiency, presenting with disseminated *M. fortuitum* and *M. avium* complex (MAC) infections. The phenotype was caused by a homozygous recessive deletion 278del2 in the IFN γ R2 coding region, resulting in a premature stop codon upstream from the segment encoding the transmembrane domain. No cell surface expression of IFN γ R2 was detected in this patient.

1.4.1.4 Partial IFN γ R2 Deficiency

Partial IFN γ R2 deficiency has been described, presents with a milder phenotype and mutations are autosomal recessive. Doffinger *et al.* 2000a described the first partial IFN γ R2 deficiency in a 20 year old patient with a history of BCG and *M. abscessus* infection. This C340T/R114C mutation did not prevent cell surface expression of the receptor.

IFN γ R2 defects are less common than the IFN γ R1 defects. This may be due to a diagnosis bias, as reagents for flow cytometry and immunoblotting for IFN γ R2 have been inferior to those of IFN γ R1 (Holland 2001).

Additional detailed IFN γ R2 gene mutations are listed in table 1.3.

Mutation	Phenotype	Effect of mutation	Country of origin	Infections	Reference
278del2	Complete recessive	No detectable protein expression, no phosphorylation of STAT1	England/Portugal	<i>M. fortuitum</i> , <i>M. avium</i> complex	Dorman and Holland 1998
C340T/R114C	Partial recessive	Reduced cell surface expression of non functional receptor	Portugal, living in France	Disseminated BCG, <i>M. abscessus</i>	Doffinger <i>et al.</i> 2000a, Doffinger <i>et al.</i> 2001
791delG in 2 siblings	Complete recessive	Truncated protein lacking intracellular domain	Qatari	<i>M. abscessus</i> , <i>M. avium</i>	Rosenzweig <i>et al.</i> 2004
C503A/T168N	Complete recessive	Glycosylation detrimental, normal protein expression	Iran (1), Saudi Arabia (2)	<i>M. avium</i> , disseminated BCG, <i>M. fortuitum</i>	Vogt <i>et al.</i> 2005, Mansoori <i>et al.</i> 2005
663del27	Complete recessive	Glycosylation detrimental, misfolded proteins	Austria	<i>M. avium</i>	Vogt <i>et al.</i> 2005
382-387dup	Complete recessive	No detectable protein expression, misfolded protein	Not reported	<i>M. avium</i> , disseminated BCG	Vogt <i>et al.</i> 2008
949delTG	Complete recessive	Loss of function	Japan	<i>M. avium</i> , <i>M. fortuitum</i> , <i>M. porcium</i> .	Toyoda <i>et al.</i> 2010
G227R	Partial recessive	Reduced protein expression of non functional receptor	Not reported	Not reported	De Paus <i>et al.</i> 2011, Kilic <i>et al.</i> 2012

Table 1.3: Reported IFN γ R2 Deficiencies

1.4.2 IFN γ Deficiency

Despite its critical role in intracellular infection, to date an MSMD phenotype due to a mutation in the human IFN γ gene has not been

described. There exists the possibility that IFN γ deficiency could be extremely rare or fatal (Ottenhof *et al.* 2002). Acquired IFN γ deficiency however, has been reported.

1.4.2.1 874T/A SNP

A functional polymorphism 874T/A has been identified in the IFN γ gene. It correlates with decreased IFN γ production, but has not been conclusively linked to susceptibility to *M. tb* infection.

1.4.2.2 Autoantibody mediated IFN γ Deficiency

Anti-IFN γ autoantibodies may be present naturally in certain clinical conditions, *eg* viral infections (Caruso *et al.* 1990) and African trypanosomiasis (Bonfanti *et al.* 1995, Madariaga *et al.* 1998).

There have however been reports of anti-IFN γ autoantibodies causing acquired IFN γ deficiency, resulting in infections with NTM.

In a report by Hoflich *et al.* 2004, a previously healthy female adult from Thailand presented with *M. chelonae* and *Burkholderia coccovenans* infections. Results of whole blood stimulation with concanavalin A showed no detection of IFN γ production, but stimulation of PBMCs showed normal levels of IFN γ production, suggesting an inhibitory factor in the patient's plasma. Plasma IgG depletion by protein A removed the anti IFN γ activity and the autoantibody was identified as an IgG4 isotype but the patient died before the inhibitory factor was identified.

Doffinger *et al.* 2004 also reported an adult Filipino male with an autoantibody to IFN γ . This patient had disseminated *M. tb* and *M. chelonae* infections. Passing the patient's serum through an affinity column coupled to IFN γ , removed the ability of the serum to inhibit the

biological activity of the IFN γ . IgG antibodies specific for IFN γ , were eluted from the column.

Three adult cases of acquired predisposition to mycobacterial disease due to autoantibodies to IFN γ were reported by Kampmann *et al.* 2005. Two females from the United Kingdom and one South African male affected with severe NTM infections, were described. The two females had MAI, *M. fortuitum* and *Aspergillus* infections and the male had an MAI infection in the vertebra. No IFN γ was detectable in patients' plasma following stimulated whole blood assays. However in the absence of autologous plasma, patients' washed PBMCs produced IFN γ in response to stimulation with relevant antigens. An IFN γ neutralizing factor, shown to be an autoantibody against IFN γ was isolated from patients' plasma by using affinity chromatography, surface-enhanced laser desorption/ionization (SELDI) mass spectrometry.

High density cDNA microarray analysis showed that the anti-IFN γ antibodies were functional and blocked induction of IFN γ inducible genes. Once the correct diagnosis had been made, the patients were successfully treated.

A study by Patel *et al.* 2005 screened 35 patients with either disseminated or pulmonary NTM infection for whom no molecular defect was known. They identified functional anti-IFN γ autoantibodies in a group of six East Asian women. These autoantibodies were high titre, shown to block binding of native human IFN γ and inhibited IFN γ dependent up-regulation of TNF α and IL12 production.

These studies show that acquired defects in the IFN γ pathway may also cause unusual susceptibility to intracellular pathogens in patients without genetically determined immunological defects.

1.4.2.3 Genetic association with autoantibody mediated IFN γ deficiency

Anti-IFN γ autoantibodies seen in a group of Taiwanese adults with NTM infections, have since been shown to be associated with two HLA-II alleles, HLA-DRB1*16:02 and HLA-DQB1*05:02 (Chi *et al.* 2013, Puel and Casanova 2013). This is the first genetic evidence of anti-IFN γ autoantibody production in humans being the cause of MSMD.

1.4.3 IL12R β 1 Deficiency

IL12R β 1 deficiency is the most common form of MSMD (de Beaucoudrey *et al.* 2010).

Mutations in the gene encoding the β 1 subunit of the IL12 receptor were first identified in seven patients from six families with a mild phenotype (Altare *et al.* 1998c, de Jong *et al.* 1998).

Impaired IFN γ secretion is also probably responsible for MSMD in patients with IL12R β 1 deficiency and residual IL12 independent IFN γ mediated immunity probably accounts for the milder clinical and histological phenotype.

Because the IL23R contains the IL12R β 1 subunit, it is possible that patients with IL12R β 1 deficiency probably also have IL23R deficiency (Parham *et al.* 2002). As IL12R β 1 deficiency may impair both IL12 and IL23 signaling, Hoeve *et al.* 2003 investigated this by studying IL23 responses of four IL12R β 1 deficient patients. In controls, IL23 promoted IFN γ produced by CD3 $^{+}$ and CD8 $^{+}$ T cells, but IL12R β 1

deficient T cells lacked IL23 induced IFN γ secretion, showing that IL23 cooperates with IL12 and IL18 in promoting IFN γ and that IL12R β 1 deficiency may result in defective IL12 and IL23 mediated immunity.

The IL12R β 1 deficiency has been renamed as IL12- and IL23R β 1 chain deficiency (Al-Herz *et al.* 2011) and this notation will be used forthwith.

The IL12- and IL23R β 1 mutation database has been updated and described (van de Vosse *et al.* 2013).

Additional detailed IL12- and IL23 β 1 gene mutations are listed in table 1.4.

Mutation	Phenotype	Effect of mutation	Country of origin	Infections	Reference
A913T/K305X	Complete recessive	Premature stop codon, no detectable protein expression	Morocco	Disseminated BCG, <i>S. enteriditis</i>	Altare <i>et al.</i> 1998c, Boisson-Dupuis <i>et al.</i> 2011
781+G→C splice site	Complete recessive	No detectable protein expression	Turkey	Disseminated BCG, <i>S. enteriditis</i>	Altare <i>et al.</i> 1998c, Tanir <i>et al.</i> 2006
A641G/Q214R	Conflicting reports	No detectable protein expression (Altare <i>et al.</i> 1998c), non pathogenic (Sakai <i>et al.</i> 2001, Elloumii-Zghal <i>et al.</i> 2002)	Cyprus, Japan	<i>M. avium</i> , <i>S. enteriditis</i>	Altare <i>et al.</i> 1998c, Sakai <i>et al.</i> 2001, Elloumii-Zghal <i>et al.</i> 2002, Bassumy <i>et al.</i> 2003
C94T/Q32X	Complete recessive	Premature stop codon, no detectable protein expression	Netherlands, France, Belgium	<i>M. avium</i> , <i>S. paratyphi</i>	de Jong <i>et al.</i> 1998, Fieschi <i>et al.</i> 2003, de Boer <i>et al.</i> 2008

C1126T/Q376X	Complete recessive	Premature stop codon	Netherlands, France	<i>Salmonella</i> type B, <i>S. dublin</i>	de Jong <i>et al.</i> 1998, Fieschi <i>et al.</i> 2003
del409-549	Complete recessive	Premature stop codon	Turkey	Disseminated BCG, <i>S. typhi</i>	de Jong <i>et al.</i> 1998
C701T/R213W as reported by Sakai <i>et al.</i> 2001	Complete recessive	Premature stop codon, no detectable protein expression	Japan, Morocco	Disseminated <i>M. avium</i>	Sakai <i>et al.</i> 2001
C637T/R213W as reported by Altare <i>et al.</i> 2001	Complete recessive	Premature stop codon, no detectable protein expression	Morocco	Disseminated BCG, abdominal tuberculosis	Altare <i>et al.</i> 2001
G518C/R173P	Complete recessive	No detectable protein expression	Turkey	<i>M. fortuitum-chelonae</i> , <i>S. enteritidis</i> , <i>S. paratyphi</i> , <i>S. typhi</i> , cutaneous leukocytoclastic vasculitis	Aksu <i>et al.</i> 2001, Fieschi <i>et al.</i> 2003, Kutukculer <i>et al.</i> 2006, Sanal <i>et al.</i> 2006, de Boer <i>et al.</i> 2008
550-2A→G splice site	Complete recessive	Skipping of exons 5 and 6, premature stop codon, no detectable protein expression	Tunisia	Disseminated BCG	Elloumi-Zghal <i>et al.</i> 2002
64+5G→A splice site	Complete recessive	Deletion of exon 2, premature stop codon, no detectable protein expression	Tunisia	Disseminated BCG	Elloumi-Zghal <i>et al.</i> 2002
T656C/C198R	Partial recessive	No detectable protein expression, but intracellular protein measured	Turkey	Disseminated BCG	Lichtenauer-Kaligis <i>et al.</i> 2003, van de Vosse and Ottenhof 2006
531-547del	Complete recessive	Premature stop codon	Turkey	Disseminated BCG	Lichtenauer-Kaligis <i>et al.</i> 2003
846+1G→A	Complete recessive	Premature stop codon	Turkey	Disseminated BCG	Lichtenauer-Kaligis <i>et al.</i> 2003

621-627delins8	Complete recessive	Premature stop codon	Turkey	Disseminated BCG	Lichtenauer-Kaligis <i>et al.</i> 2003
557-563delins8	Complete recessive	Not reported	Turkey	<i>S. enteriditi, Candida</i>	Fieschi <i>et al.</i> 2003
Y367C	Complete recessive	Not reported	Cameroon	<i>M. avium, M. triplex, S. dublin</i>	Fieschi <i>et al.</i> 2003
1190-1G→A	Complete recessive	Not reported	Saudi Arabia	<i>Salmonella</i> species	Fieschi <i>et al.</i> 2003
1791+2T→G	Complete recessive	No detectable protein expression	Iran, Sri Lanka, Spain, Turkey, China, Mexico	Extraintestinal salmonellosis, <i>M. avium, S. enteriditis</i> , disseminated BCG, oral candidiasis, <i>Klebsiella, Candida albicans</i>	Caragol <i>et al.</i> 2003, Fieschi <i>et al.</i> 2003, Lee <i>et al.</i> 2008, Pedraza <i>et al.</i> 2010, Pedraza-Sanchez <i>et al.</i> 2010b
S321X	Complete recessive	Not reported	Pakistan	<i>S. enteriditis</i>	Fieschi <i>et al.</i> 2003
Q32X and 1623_1624delins TT compound heterozygote	Complete recessive	Not reported	France	Not reported	Fieschi <i>et al.</i> 2003
1745_1746insCA and 1483+182-1619-1073del compound heterozygote	Complete recessive	Not reported	France	<i>S. dublin</i>	Fieschi <i>et al.</i> 2003
549+2T→C	Complete recessive	Not reported	Bosnia-Herzegovina	Not reported	Fieschi <i>et al.</i> 2003
1440_1447delins 16 and Q171P compound heterozygote	Complete recessive	Not reported	Slovakia	Not reported	Fieschi <i>et al.</i> 2003
1007_1008delins G and Q171P compound heterozygote	Complete recessive	Premature stop codon, expression of nonfunctional protein	Slovakia	Disseminated BCG	Fieschi <i>et al.</i> 2003, Ulrichs <i>et al.</i> 2005
L77P	Complete recessive	Not reported	Brazil	<i>S. typhi, Paracoccidioides brasiliensis</i>	Fieschi <i>et al.</i> 2003, de Moraes Vasconcelos <i>et al.</i> 2005
C186S and ataxia telangiectasia	Complete recessive	Not reported	Qatar	S. group D	Fieschi <i>et al.</i> 2003, Ehlayel <i>et al.</i> 2008
1623_1624delGC insTT/Q542X	Complete recessive	Not reported	Germany	<i>M. avium, S. enteriditis</i>	Fieschi <i>et al.</i> 2003, Haerynck <i>et al.</i> 2008

783+1G→A	Complete recessive	Skipping of exon 8 or exons 5 and 8, no detectable protein expression	Turkey	<i>S. enteriditis</i>	Fieschi <i>et al.</i> 2003, Tanir <i>et al.</i> 2006, Sanal <i>et al.</i> 2006
T→G splice acceptor site	Complete recessive	Skipping of exon 15, premature stop codon, no detectable protein expression	Iran	Disseminated <i>S. enteriditis</i>	Cleary <i>et al.</i> 2003
700+362_1619-944del (12165del)	Complete recessive	Reduced expression of nonfunctional protein, deletion of exons 8-13	Israel Bedouin	<i>S. group D bacteremia, M. avium</i>	Staretz-Haham <i>et al.</i> 2003, Fieschi <i>et al.</i> 2004, Scheuerman <i>et al.</i> 2007
1021+1G→C	Complete recessive	skipping of exon 9	Turkey	Abdominal tuberculosis	Ozbek <i>et al.</i> 2005
Q171P and 1440_1147del16	Complete recessive	Premature stop codon, no expression of protein	Slovakia	Disseminated BCG	Ulrichs <i>et al.</i> 2005
G696C/R211P	Complete recessive	No protein expression	Taiwan	<i>Salmonella</i> species	Filipe-Santos <i>et al.</i> 2006b, de Beaucoudrey <i>et al.</i> 2010
711insC	Complete recessive	Not reported	Turkey	Disseminated BCG	Tanir <i>et al.</i> 2006
628-644dup	Complete recessive	Not reported	Turkey	Disseminated BCG, <i>Salmonella</i> bacteremia	Tanir <i>et al.</i> 2006
C670T/R175W	Complete recessive	Not reported	Turkey	<i>Salmonella</i> bacteremia	Ozen <i>et al.</i> 2006
1623_1624insTT	Complete recessive	Not reported	Cyprus, Argentina	Not reported	Filipe-Santos <i>et al.</i> 2006b, de Beaucoudrey <i>et al.</i> 2010
1483+182_1619-1073del	Complete recessive	Not reported	Not reported	Not reported	Filipe-Santos <i>et al.</i> 2006
C198R	Complete recessive	Not reported	Not reported	Not reported	Filipe-Santos <i>et al.</i> 2006b
R211P	Complete recessive	Not reported	Taiwan	Not reported	Filipe-Santos <i>et al.</i> 2006b, de Beaucoudrey <i>et al.</i> 2010
S321X	Complete recessive	Not reported	Pakistan	<i>S. enteriditis</i>	Filipe-Santos <i>et al.</i> 2006b,

467-483del17	Complete recessive	Deletion of exon 5, premature stop codon	Turkey	S. group D	Sanal <i>et al.</i> 2006, Filipe-Santos <i>et al.</i> 2006b
C1520T/R486X	Complete recessive	Premature stop codon, no detectable protein expression	Japan, Mexico, Turkey	Disseminated BCG, candida sepsis, Klebsiella	Asilsoy <i>et al.</i> 2007, Pedraza <i>et al.</i> 2010a, de Beaucoudrey <i>et al.</i> 2010
65-68del4 and 1623_1624delGC insTT compound heterozygote	Complete recessive	Premature stop codon, truncated protein, no detectable protein expression	Belgium	M. avium	Haerynck <i>et al.</i> 2008
C853T/Q285X	Complete recessive	Not reported	China	Disseminated BCG	Lee <i>et al.</i> 2008
G696T/A211P	Complete recessive	No detectable protein expression	Taiwan	<i>S. enteritidis</i> D	Lee <i>et al.</i> 2009
1623_1624delins TT	Complete recessive	Argentina	Not reported	Not reported	Yancoski <i>et al.</i> 2009, de Beaucoudrey <i>et al.</i> 2010
1745_1746delins CA/C196Y and 1483+182_1619-1073del compound heterozygote	Complete recessive	Not reported	France	<i>Salmonella</i> species	de Beaucoudrey <i>et al.</i> 2010
1369T+1623_1624delinsTT compound heterozygote	Complete recessive	Not reported	Poland, Saudi Arabia	Not reported	de Beaucoudrey <i>et al.</i> 2010
Y88X	Complete recessive	Not reported	Saudi Arabia	<i>Citrobacter freundii</i>	de Beaucoudrey <i>et al.</i> 2010
983-999del and R173W compound heterozygote	Complete recessive	Not reported	Brazil	Not reported	de Beaucoudrey <i>et al.</i> 2010
R173W	Complete recessive	Not reported	Venezuela, Poland, Iran	<i>Salmonella</i> species, <i>Candida</i>	de Beaucoudrey <i>et al.</i> 2010, Boisson-DuPuis <i>et al.</i> 2011
1189+2T→A and 1791+2T→G compound heterozygote	Complete recessive	Not reported	Ukraine	Salmonella	de Beaucoudrey <i>et al.</i> 2010
R521X	Complete recessive	Not reported	India, Iran	Histoplasmosis	de Beaucoudrey <i>et al.</i> 2010

169del11 and C62G compound heterozygote	Complete recessive	Not reported	Chile	Not reported	de Beaucoudrey <i>et al.</i> 2010
Q285X	Complete recessive	Not reported	China	Not reported	de Beaucoudrey <i>et al.</i> 2010
E67X and 1623_1624delins TT compound heterozygote	Complete recessive	Not reported	Argentina	Not reported	de Beaucoudrey <i>et al.</i> 2010
E480X	Complete recessive	Not reported	Ukraine	<i>Salmonella</i> species	de Beaucoudrey <i>et al.</i> 2010
R175W	Complete recessive	Not reported	Turkey	<i>Salmonella</i> species	de Beaucoudrey <i>et al.</i> 2010
1765delG	Complete recessive	Not reported	French West Indies	<i>Salmonella</i> species, mycobacteria species	de Beaucoudrey <i>et al.</i> 2010
C198R	Complete recessive	Not reported	Turkey	<i>Salmonella</i> species	de Beaucoudrey <i>et al.</i> 2010
64+2T→G	Complete recessive	Not reported	Turkey	<i>Candida</i>	de Beaucoudrey <i>et al.</i> 2010
1425delC	Complete recessive	Not reported	Turkey	<i>Salmonella</i> species, <i>Candida</i>	de Beaucoudrey <i>et al.</i> 2010
G569D	Complete recessive	Not reported	Iran	<i>M. tb</i>	de Beaucoudrey <i>et al.</i> 2010
W531X	Complete recessive	Not reported	Argentina	Not reported	de Beaucoudrey <i>et al.</i> 2010
1369T and R211P compound heterozygote	Complete recessive	Not reported	China	Not reported	de Beaucoudrey <i>et al.</i> 2010
1386_1387delGT	Complete recessive	Not reported	Tunisia	Not reported	de Beaucoudrey <i>et al.</i> 2010
1623_1624insTT and del exon 4 compound heterozygote	Complete recessive	Not reported	Argentina	Not reported	de Beaucoudrey <i>et al.</i> 2010
580+1G→A	Complete recessive	Not reported	Iran	Not reported	de Beaucoudrey <i>et al.</i> 2010
64+1G→T	Complete recessive	No detectable protein expression	Turkey	Disseminated BCG, oral candidiasis	Aytekin <i>et al.</i> 2011
1336delC	Complete recessive	No detectable protein expression	Saudi Arabia	Disseminated BCG	Alangare <i>et al.</i> 2011

355delT	Complete recessive	Not reported	Not reported	MDR TB	Tabarsi <i>et al.</i> 2011
C19602T/R283X and IgA deficiency	Complete recessive	No detectable protein expression	Faroe Islands	Low virulence mycobacteria	Schejbel <i>et al.</i> 2011

Table 1.4: Reported IL12- and IL23R β 1 deficiencies

1.4.4 IL12R β 2 Deficiency

Kondo *et al.* 2001a and Kondo *et al.* 2001b reported reduced IFN γ production in response to stimulation with IL12 in atopic Japanese subjects with mutations in the IL12R β 2 gene but no associations were found with mycobacterial disease. Bassumy *et al.* 2003 did a study looking at associations between type I diabetes and asthma and IL12R β 2 gene mutations in the Japanese population and again, no associations were found with mycobacterial disease.

A possible reason why no IL12R β 2 defects have been found to be associated with mycobacterial disease may be because IL23R signaling can compensate for an IL12R β 2 defect (van de Vosse *et al.* 2004).

1.4.5 IL12p40 Deficiency

In 1998 a child born to consanguineous Pakistani parents presented with a mild histopathological and clinical phenotype and a recessive mutation in the IL12p40 gene was reported. This first mutation was reported as a homozygous frameshift deletion of 4.4 kilobases (482del373), encompassing two coding exons 5 and 6 (Altare *et al.* 1998a, Altare *et al.* 1998d).

The same 482del373 mutation was also found in an Indian family in which it caused fatal disseminated BCG infection in one child and repeated infections of *Salmonella enteritidis* in a second affected child (Picard *et al.* 2002). The mutation was reported as a 4.6 kilobase

deletion designated 482+82_856-854 del (deletion from 482+82 in intron 4 donor site and 856 or 854 in exon 6) resulting in a loss of 167 of original 328 amino acids and addition of 45 new amino acids.

These two descriptions differ slightly in the two literature reports, but have been quoted as the same deletion.

Mycobacterial infections in these deficiencies occur primarily because IFN γ mediated immunity is impaired and residual IL12 dependent secretion of IFN γ is probably responsible for the milder clinical phenotype. Patients with mutations in the IL12p40 gene are susceptible to infections with *Salmonella* species.

Additional detailed IL12p40 gene mutations are listed in table 1.5.

Mutation	Phenotype	Effect of mutation	Country of origin	Infections	Reference
315insA in 4 families	Complete recessive	Impaired secretion of IL12	Saudi Arabia	Disseminated BCG, <i>Salmonella</i> species	Picard <i>et al.</i> 2002
482+82_856-854del in 2 families	Complete recessive	Impaired secretion of IL12	Pakistan, India	Disseminated BCG, <i>Salmonella</i> species	Altare <i>et al.</i> 1998a, Altare <i>et al.</i> 1998d, Picard <i>et al.</i> 2002
297del8 in 2 families	Complete recessive	Premature stop codon	Tunisia	Disseminated BCG	Elloumi-Zghal <i>et al.</i> 2002
526-528delCT	Complete recessive	Impaired secretion of IL12	Iran	Disseminated BCG	Mansouri <i>et al.</i> 2005
35del10	Complete recessive	Not reported	Saudi Arabia	Not reported	Prando <i>et al.</i> 2013
909insA	Complete recessive	Preamature stop codon at 307	Iran	Not reported	Prando <i>et al.</i> 2013
W60X	Complete recessive	Not reported	Not reported	Not reported	Prando <i>et al.</i> 2013
697+2T→C	Complete recessive	Deletion of exon 6, terminated protein	Iran	Not reported	Prando <i>et al.</i> 2013
697+5G→A	Complete recessive	Deletion of exon 6, terminated protein	India	Not reported	Prando <i>et al.</i> 2013

Table 1.5: Reported IL12p40 deficiencies

1.4.6 STAT1 Deficiencies

1.4.6.1 Partial STAT1 Deficiency

The first STAT1 defect T2116C/L706S reported was a partial deficiency (Dupuis *et al.* in 2001), with a clinical phenotype similar to that of the partial IFN γ R deficiencies (Jouanguy *et al.* 1997b, Jouanguy *et al.* 1999, Doffinger *et al.* 2000a). This type of deficiency results in impaired anti-mycobacterial immunity but unimpaired anti-viral immunity.

1.4.6.2 Complete STAT1 Deficiency

Complete recessive STAT1 deficiency results in impairment of both IFN γ mediated anti-mycobacterial immunity and IFN α mediated anti-viral immunity, resulting in a severe phenotype.

In 2003, the first cases of complete STAT1 deficiency were reported in two unrelated Saudi Arabian infants born to consanguineous kindreds. These were a 2 nucleotide deletion at position 1754, 1755, 1756 or 1757 arbitrarily designated 1757-1758delAG, generating a premature stop codon at position 603 and a homozygous nucleotide substitution of T to C in exon 20, resulting in an amino acid change L600P. The parents of both infants were healthy and heterozygous for each mutation (Dupuis *et al.* 2003).

Additional detailed STAT1 gene mutations are listed in table 1.6.

Mutation	Phenotype	Effect of mutation	Country of origin	Infections	Reference
T2116C/L706S	Partial dominant for GAF activation, recessive for ISGF3 activation	Reduced tyrosine phosphorylation Impaired anti-mycobacterial immunity	France mother and daughter, USA	Disseminated BCG, <i>M.avium</i>	Dupuis <i>et al.</i> 2001
1757-1758delAG	Complete recessive	No DNA activation, no GAF or ISG3 activation, impaired anti-mycobacterial and anti-viral immunity	Saudi Arabia	Disseminated BCG, viral infections	Dupuis <i>et al.</i> 2003
L600P	Complete recessive	No DNA activation, no GAF or ISG3 activation, impaired anti-mycobacterial and anti-viral immunity	Saudi Arabia	Disseminated BCG, viral infections	Dupuis <i>et al.</i> 2003
1928insA	Complete recessive	Reduced phosphorylation, no protein expression, no GAS and ISRE protein expression, impaired anti-mycobacterial and anti-viral immunity	Pakistan	Disseminated BCG, viral infections	Chapgier <i>et al.</i> 2006a
G958C/E320Q	Partial dominant for GAF, recessive for ISG3	No DNA binding, impaired anti-mycobacterial immunity	Germany	Disseminated BCG	Chapgier <i>et al.</i> 2006b
G1389T/Q463H	Partial dominant for GAF activation, recessive for ISG3 activation	No DNA binding, impaired anti-mycobacterial immunity	Germany	<i>M. avium</i>	Chapgier <i>et al.</i> 2006b
G372C splice site	Complete recessive	Skipping of exon 3, reduced phosphorylation, no DNA binding, expression of lower MW protein, functional impact on NK cells, impaired anti-mycobacterial and anti-viral immunity	Pakistan living in Italy	<i>M. kansasii</i> , viral infections	Vairo <i>et al.</i> 2011

Table 1.6: Reported STAT1 deficiencies

The molecular analyses of these genetic defects identified from 1996 onwards, have led to a better understanding of the importance of the IFN γ pathway in protection against TB. Identification of new genetic

defects is ongoing, but the foundation of understanding was laid from these early reports resulting in important diagnostic and therapeutic implications.

Reference sources for mutations were www.ncbi.nlm.nih.gov/pubmed, www.google.com, UCT libraries database and the time frame was to July 2013.

1.5 MSMD Caused by Mutations in Genes Outside of the IL12/IL23/IFN γ Pathway

Mutations in certain genes outside of the IL12/IL23/IFN γ pathway can also cause MSMD.

There were early reports of X-linked modes of inheritance associated with mycobacterial disease (Nedorost *et al.* 1991, Holland *et al.* 1994, Frucht and Holland 1996, Frucht *et al.* 1999).

1.5.1 X linked NF κ B essential modulator (NEMO) deficiency (XR-MSMD-1)

Nuclear factor kappa B (NF κ B) is a collective name for a heterologous group of dimeric transcription factors composed of members of the Rel family of DNA binding proteins that recognize a common sequence motif (Karin and Ben-Neriah 2000).

The NF κ B dimers are ubiquitously expressed and their interaction with the inhibitor of NF κ B (I κ B) regulates NF κ B activity. The I κ Bs in mammals include I κ B α , I κ B β and I κ B ϵ . The I κ B kinase complex IKK regulates I κ B phosphorylation resulting in its ubiquitination and proteasome degradation, releasing NF κ B into the nucleus to initiate the process of gene transcription.

IKK is composed of 3 subunits, IKK α and IKK β acting as catalytic components and IKK γ , also known as NF κ B essential modulator (NEMO) which acts as the structural scaffolding supporting the IKK complex (Karin *et al.* 2000, Rosenzweig and Holland 2005). The gene that encodes NEMO is required for activation of NF κ B transcription. It is located on the X chromosome and has 10 exons. The NEMO protein is a 419 amino acid regulatory protein (Aradhya *et al.* 2001).

X – linked recessive NEMO mutations cause anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID), a developmental and immunologic disorder (Zonana *et al.* 2000). NEMO deficiency can impair B cell function, T cell development and survival, NK cell cytotoxicity and TLR immune responses. Patients with NEMO mutations can also have increased susceptibility to mycobacterial disease (Hanson *et al.* 2008) as well as other infections (Orange *et al.* 2004).

In 2006, an X-linked recessive MSMD syndrome caused by NEMO mutations was reported (Filipe-Santos *et al.* 2006). This group reported on mutations in the leucine zipper domain of the NEMO gene in three unrelated kindreds from the USA, France and Germany, with this syndrome. Mutations were found in the coding regions of the NEMO genes in all three probands. One NEMO mutation identified in the American kindred was A944C/E315A and a second mutation G956A/319Q was identified in both the French and German kindreds. These two mutations did not affect NF κ B activation in response to most of the classical activators tested, but were found to selectively impair the CD40 triggered and NF κ B/c-Rel-mediated induction of IL12 production by monocytes and dendritic cells, accounting for the susceptibility to mycobacterial infections.

Impaired secretion of IL12 by PHA stimulated monocytes and T cells was observed in patients with the NEMO mutations, possibly affecting CD40 signaling within monocytes, resulting in low levels of IL12 secretion upon T cell stimulation.

Imamura *et al.* 2011 reported a Japanese child with disseminated BCG infection caused by a C913G/D311E NEMO mutation. This mutation was located in the NEMO ubiquitin-binding domain. Flow cytometry showed that NEMO expression was not detected in the patient's B cells, monocytes or NK cells as measured by expression on CD19+, CD14+ or CD56+ cells by anti NEMO monoclonal antibodies.

B cell responses to CD40L as measured by flow cytometry were found to be severely impaired. Both IFN γ secretion induced by stimulation with IL18 and IL12 and TNF α secretion in response to LPS stimulation in the absence or presence of IFN γ were severely impaired.

1.5.2 XR-MSMD-2 deficiency

Bustamante *et al.* 2007b also studied a large kindred with four male maternal relatives affected with mycobacterial disease. Three of the subjects had recurrent disease after BCG vaccination and the fourth had recurrent TB. Screening by whole blood assays excluded all known forms of autosomal recessive and dominant disorders of the IL12/IL23-IFN γ pathway. There was abnormal CD40-dependent IL12 production by monocytes in the presence of autologous and heterologous PHA activated T cells.

In this kindred, no NEMO mutations were found, NEMO expression was normal in an EBV-B cell line and fresh blood cells as shown by Western blotting and flow cytometry showed normal NEMO expression on all leukocyte subsets.

This work excluded the NEMO gene as the cause of disease using clinical observations (lack of ectodermal dysplasia), immunological results (normal IL12 production by monocytes), genetic analysis (normal sequence of NEMO coding region), biochemical data (normal NEMO protein production) and linkage analysis (NEMO encompassing region excluded by X scan).

As a result of this finding, a new form of X-linked recessive MSMD, designated XR-MSMD-2 was reported, but the genetic defect was not identified.

1.5.3 X-linked Chronic Granulomatous Disease (CGD) (CYBB deficiency) predisposing to TB disease

Patients with CGD are susceptible to TB and BCG complications. About 70% of cases are transmitted by X-linked mode of inheritance, arising from mutations in the gp91-^{phox} subunit of the phagocyte NADPH oxidase, the protein encoded by the CYBB gene, localized on chromosome Xp21.1 (Bustamante *et al.* 2007a, Lee *et al.* 2008).

MSMD caused by X-linked Chronic Granulomatous Disease (CGD) has been reported. Two kindreds in which male adults with previously unknown mutations in the CYBB gene, resulting in an impaired respiratory burst in macrophages, but not monocytes or granulocytes, developed MSMD (Bustamante *et al.* 2011). The mutations identified in the two kindreds were A231C/Q231P and A178C/T178P in the gp91-^{phox} subunit. Affected males in both kindreds were heterozygous for the mutations.

1.5.4 IRF-8 deficiency

MSMD due to interferon regulatory factor 8 (IRF8), a protein essential for the development of dendritic cells and the differentiation of

macrophages and granulocytes has been documented. Mutations in the IRF8 gene, localized on chromosome 16q24.1, impair IL12 secretion by monocytes and dendritic cells. One case of autosomal recessive IRF8 deficiency caused by a K108E mutation is associated with severe disease and complete depletion of monocytes and dendritic cells and two unrelated cases of autosomal dominant IRF8 deficiency caused by a T80A mutation, presenting with a milder phenotype, have been reported (Hambleton *et al.* 2011, Salem *et al.* 2103).

1.5.5 ISG15 deficiency

MSMD due to mutations in the ISG15 gene, encoding an IFN α/β inducible, ubiquitin-like intracellular protein, have been reported. Two unrelated patients with homozygous mutations G379T/Q127Q and 336_337insG had impaired but not abolished IFN γ immunity and mild MSMD (Bogunovic *et al.* 2012).

1.6 MSMD Phenotype

MSMD is a phenotypically heterogeneous syndrome and many of these patients present with a variable array of atypical clinical symptoms (Dupuis *et al.* 2000). The disorders are genetically different but immunologically similar, impaired IFN γ mediated immunity being the common pathogenic mechanism accounting for the mycobacterial infection.

1.6.1 Clinical features

Individuals with impaired cell mediated immunity due to MSMD exhibit increased susceptibility to opportunistic infections caused by NTM, BCG and *Salmonella* species, *M. avium* being the most common opportunistic infection (Casanova *et al.* 1995, Kumararatne 1997,

Doffinger *et al.* 1999). *Salmonella* infection is more common among individuals with IL12 or IL12- and IL23R β 1 receptor deficiency (de Jong *et al.* 1998, Altare *et al.* 1998c) while this infection occurred in a minority of patients with IFN γ receptor deficiency (Jouanguy *et al.* 1996, Casanova *et al.* 1996, Newport *et al.* 1996, Haverkamp *et al.* 2006). Additional infectious organisms found in MSMD patients are listed in tables 1.2-1.6.

There is correlation between the genotype (null or mild mutation), the cellular phenotype (complete or partial defect), the histological phenotype (immature or mature granulomas) and clinical phenotype (poor or favourable outcome).

Children with lepromatous like granulomas that are poorly delimited, multibacillary with no epithelioid or giant cells generally die of overwhelming infection (Emile *et al.* 1997).

Complete IFN γ R1 and IFN γ R2 deficiencies predispose patients to this type of overwhelming infection with impaired granuloma formation in infancy or early childhood.

Children presenting with tuberculoid granulomas that are well delimited and paucibacillary with epithelioid and giant cells have a more favourable outcome and are associated with survival.

These are seen in patients with partial IFN γ R1, partial IFN γ R2, partial STAT1, IL12p40 and IL12R β 1 deficiencies predisposing them to curable infection with mature granulomas at various ages.

The categories of mutations causing MSMD can result in defective response to IFN γ , defective response to IL12, defective production of

IL12, defective production of IFN γ , resulting in impairment of anti-mycobacterial immunity and anti-viral immunity.

These are summarized in table 1.7.

Deficiency	Phenotype	Laboratory diagnostics
IFNγR1		
Complete recessive	Severe, early onset NTM, Salmonella infections, disseminated BCG	No response to IFN γ in WBAs*. High serum IFN γ levels. Absence of receptor expression or overexpression of nonfunctional receptor seen by flow cytometry.
Partial recessive	Milder, later onset NTM infection, disseminated BCG	Partial response to IFN γ in WBAs*. Response can be increased by using high concentrations of IFN γ .
Partial dominant	Milder, later onset NTM, osteomyelitis common	Partial response to IFN γ in WBAs*. Response can be increased by using high concentrations of IFN γ .
IFNγR2		
Complete autosomal recessive	Severe, early onset NTM, Salmonella infections, disseminated BCG	No response to IFN γ in WBAs*.
Partial autosomal recessive	Milder, later onset NTM infection, disseminated BCG	Partial response to IFN γ in WBAs*. Response can be increased by using high concentrations of IFN γ .
IFNγ		
Acquired autoantibodies	Severe, NTM infections, disseminated BCG.	Undetectable IFN γ in WBAs*. In absence of autologous plasma, detectable, IFN γ levels secreted by PBMCs, measured in WBAs*.
IL12- and IL23Rβ1		
Complete autosomal recessive	Severe, early onset NTM infections, disseminated BCG. Salmonella infections common.	No response to IL12 in WBAs*. Absence of receptor expression seen by flow cytometry.
Partial autosomal recessive	Milder, later onset NTM infection, disseminated BCG.	Partial response to IL12 in WBAs*.
IL12p40		

Complete recessive	autosomal	Milder, NTM infections, disseminated BCG. Salmonella infections common.	Undetectable or low IL12 levels in WBAs*.
STAT1			
Complete recessive	autosomal	Severe, impaired anti-mycobacteria, impaired anti-viral immunity, disseminated BCG.	Undetectable or low IFN γ levels in WBAs*. Immunity analyzed by EMSA, nuclear binding assays.
Partial recessive	autosomal	Milder, impaired anti-mycobacterial immunity, unimpaired anti-viral immunity, disseminated BCG.	Low IFN γ levels in WBAs*. Immunity analyzed by EMSA, nuclear binding assays.
NEMO			
Partial recessive	X-linked	Disseminated BCG, <i>M. avium</i> , mycobacterial infections, <i>S. pneumonia</i> .	Impaired B cell, T cell, NK cell function, CD40-IL12 signaling. Undetectable or low IFN γ and IL12 levels in WBAs. Absence of protein detected by Western blotting.
CYBB			
Partial recessive	X-linked	Disseminated BCG, TB.	Impaired CYBB dependent NADPH respiratory burst.
IRF-8			
Partial dominant	autosomal	Mild, mycobacterial infections.	Depletion of dendritic cells, impaired, impaired IL12 production.
Complete recessive	autosomal	Severe, mycobacterial infections.	Depletion of monocytes and dendritic cells, impaired IL12 production.
ISG15			
Complete recessive	autosomal	Impaired anti-mycobacterial immunity, unimpaired anti-viral immunity.	Low IFN γ levels produced by PBMCs and NK cells in WBAs*.

Table 1.7: Phenotype and laboratory diagnostics for deficiencies causing MSMD (*WBA screening shown in figure 1.9)

When considering diagnosis of MSMD, the following clinical features, in the absence of other identifiable causes of immunodeficiency are important to note:

- Patients with disseminated or recurrent infection due to poorly pathogenic mycobacteria (BCG or NTM).
- Disseminated MAC infection is an indication of manifestation of an immunological defect (Holland 2001).
- Patients with systemic infections caused by non-typhi *Salmonella* species that are persistent and recurrent despite antibiotic treatment.
- Patients with *M. tb* infections who are treatment compliant, have drug sensitive organisms but develop recurrent or disseminated disease.
- Diagnostic clues for identifying partial dominant IFN γ R1 deficiencies can be slow healing of BCG scars, family history of infections with NTM, *Salmonella* infections and lack of response to Langerhans' Cell histiocytosis (LCH) therapy (Edgar *et al.* 2001).
- Children with Histiocytosis-X, unresponsive to treatment, or with atypical pathological features (Lammas *et al.* 2001).
- Patients with severe unexplained viral infections including CMV, HSV, Varicella zoster virus, RSV, Parainfluenza virus type 3, because a subset of patients with MSMD may manifest unexplained viral infections (Dorman *et al.* 1999).

1.6.2 Laboratory screening

Diagnosis remains challenging, but as a start a few standard assays may be done as an initial screening process. These tests include whole blood up-regulation assays followed by ELISAs to detect secretion of $\text{TNF}\alpha$, IL12 and $\text{IFN}\gamma$ cytokines and cellular responses to $\text{IFN}\gamma$ and IL12. The measurement of $\text{IFN}\gamma$ and IL12 receptors may be done by flow cytometry. This is depicted in figure 1.9.

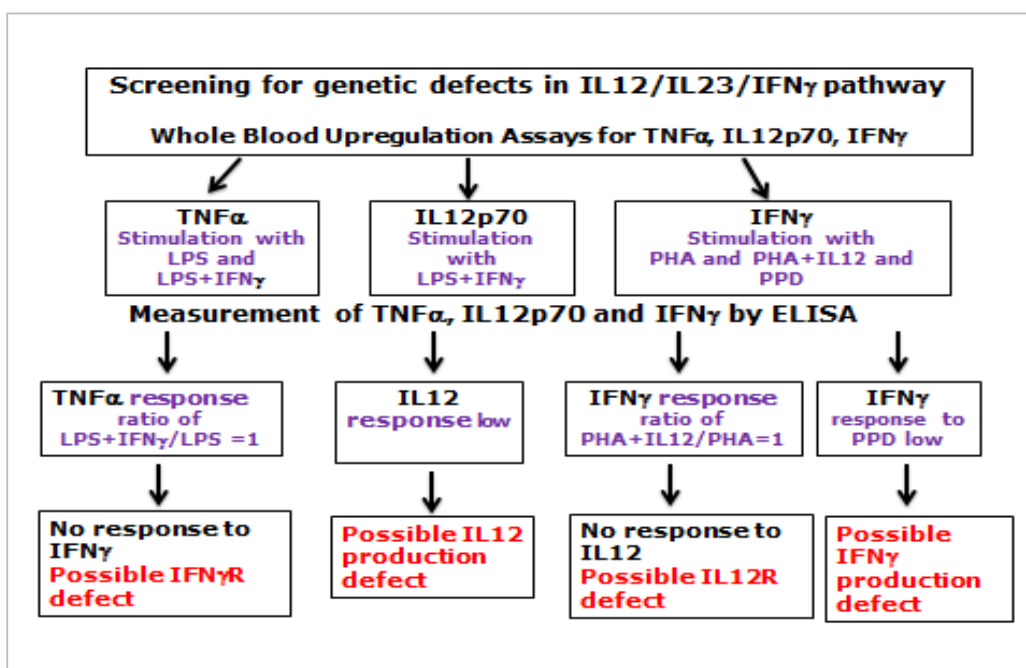


Figure 1.9: Algorithm showing laboratory screening for defects in IL12/IL23/IFN γ pathway.

These are more fully discussed in Chapter 5 (Immune testing).

The discovery of these genetic disorders has had important diagnostic and therapeutic implications with respect to bone marrow transplantation, IFN γ treatment and anti-mycobacterial drugs. A better understanding of the critical elements of these cytokine

pathways has also aided in ways to circumvent genetic defects through treatment with complementary cytokines (Holland 2000).

1.6.3 Treatment

Complete recessive IFN γ R deficiency patients have a poor prognosis, responding less well to anti-mycobacterial chemotherapy and bone marrow transplantation is challenging (Dorman *et al.* 2004). Transplant engraftment has been shown to be suboptimal in complete IFN γ R1 deficient patients, possibly due to the high levels of circulating IFN γ in these patients and active mycobacterial infection impairs success in Haematopoietic Stem Cell Transplantation (HSCT) (Roessler *et al.* 2006).

Partial deficiencies are good candidates for successful cytokine prophylaxis and therapy (Holland 2000), showing a favourable response to anti-mycobacterial chemotherapy and treatment with exogenous IFN γ . It is important to identify and describe mutations as it then becomes possible to choose appropriate treatment. For example the T832G/E278X mutation causing partial dominant IFN γ R1 deficiency allows some cell surface expression of IFN γ R1 receptors. This made it possible to administer exogenous IFN γ to flood existing functional and non-functional receptor sites during episodes of osteomyelitis to ensure immunological response from patient's cells. Dose escalation of IFN γ is necessary, possibly to achieve saturation of the nonfunctional cell surface IFN γ R1 chains. Treatment of IL12R β 1 deficient patients with high doses of IFN γ has also been shown to have positive clinical effects (Alangari *et al.* 2011).

Obtaining an accurate molecular diagnosis is therefore crucial for determining optimal treatment strategy.

1.7 Human Genetic Susceptibility to Infection with *Mycobacterium tuberculosis*

Differences in susceptibility to *M. tb* reported in different racial and ethnic groups (Stead *et al.* 1990), as well as twins studies (Comstock 1978, van der Eijk *et al.* 2007) are indicators of genetic susceptibility to TB. In support of this hypothesis was also the report of a tragic incident in Lubeck in 1923 when a single virulent viable *M. tb* strain was inadvertently used to immunize 249 infants (Smith FB 1988). Responses ranged from recovery to death from overwhelming TB disease with 173 infants surviving, arguing for a genetic basis for resistance to TB.

Major gene loci important for controlling TB infection are being identified on an ongoing basis and studies are revealing a Mendelian susceptibility to infection with *M. tb* (Baghdadi *et al.* 2013).

1.7.1 MSMD patients affected with *M. tb*

The importance of the IL12/IL23/IFN γ pathway was elucidated by the incidence of NTM infections seen in MSMD patients, but susceptibility to infection with *M. tb* was also seen.

A small subset of MSMD patients become infected solely with *M. tb* (Altare *et al.* 2001, Caragol *et al.* 2003, Ozbek *et al.* 2005, Tabarsi *et al.* 2011). Following is a description of some cases.

Altare *et al.* 2001 reported clinical heterogeneity in a family affected with IL12R β 1 deficiency. Two siblings were homozygous for the C637T/R213W mutation, preventing receptor expression and abolishing cellular responses to IL12. One sibling had disseminated BCG infection in early childhood. The second sibling was unaffected by BCG immunization, receiving 3 inoculations of live BCG, but

developed abdominal TB at the age of 18 years. Neither sibling had clinical disease caused by NTM.

There was a report of a family with IL12R β 1 deficiency affected with *M. tb* infection but no infectious diseases caused by BCG or NTM infection. Three siblings in this family with the splice site mutation 1791+2T→G showed different clinical phenotypes. One child had disseminated TB, the second had Salmonella sepsis and pulmonary TB and the third was asymptomatic (Caragol *et al.* 2003).

Ozek *et al.* 2005 reported a child with IL12R β 1 deficiency caused by the 1021+1G→C splice site mutation, affected with disseminated TB.

An adult male with IL12R β 1 deficiency caused by the 355delT mutation was diagnosed with TB resistant to isoniazid and rifampicin. The TB disseminated into lymph nodes and the colon, with a fatal outcome. This is the first report of IL12R β 1 deficiency presenting with disseminated TB in an adult (Tabarsi *et al.* 2011).

A child with inherited IL12p40 deficiency caused by the 4.6kb deletion (Altare *et al.* 1998a, Altare *et al.* 1998d, Picard *et al.* 2002) was reported to have disseminated mycobacterial disease following BCG vaccination, followed by infection with *M. tb*. (Pulickal *et al.* 2008).

Picard *et al.* 2002 reported a case of IL12p40 deficiency in a child with the 315insA mutation who developed virulent *M. tb* infection.

1.8 Genetics in *M. tb* Infection

An oligenic pattern of inheritance implies that phenotype is dependent on two or a few major genes, whereas a polygenic pattern of inheritance implies that the phenotype results from the effect of a

large number of genetic loci, each having a small effect (Alcais *et al.* 2009).

Many genes may be important in susceptibility to *M. tb* including Toll like receptors, NRAMP, IL1 β and ILRa, NEMO, Vitamin D, Vitamin D receptor, Vitamin D binding protein and NOS2A. These are discussed below.

1.8.1 Toll like receptors (TLR)s

Microbial lipoproteins are potent stimulators of IL12 production by human macrophages. Induction is mediated by the TLRs, known to be important for initiation and coordination of host response to *M. tb*. There have been many studies demonstrating the importance of TLRs with respect to TB. Following is a description of some reports.

Liu *et al.* 2006 reported that TLR activation of human macrophages up-regulates expression of the vitamin D receptor and the vitamin D-1 hydroxylase genes, resulting in production of the antimicrobial peptide cathelicidin and killing of intracellular mycobacteria. This data supports the link between TLRs and Vitamin D-mediated innate immunity which is important in mycobacterial immunity.

Ma *et al.* 2007 did a study on TLR variants that contributed to susceptibility to TB. They carried out a population-based-case-control study on 1312 adults from three ethnic groups, African Americans, European Americans and Hispanics. Results suggested that variant TLRs in TLR 1, 6, 10 contribute to human susceptibility to TB disease.

Hawn *et al.* 2007 reported that a TLR1 SNP I602S regulates the innate immune responses to extracts of mycobacteria. It has also been

reported by Yim *et al.* 2008 that shorter GT repeats in intron II of the TLR2 gene were significantly associated with TB in a Korean cohort.

Davila *et al.* 2008 carried out an association study in an Indonesian cohort and found four polymorphisms in the TLR8 gene on the X chromosome, three in the promoter region, showing evidence of association with susceptibility to TB in males. They then screened a Russian cohort for these polymorphisms and confirmed this association.

Dalgic *et al.* 2011 reported on the Arg753Gln SNP in TLR2 in a paediatric Turkish cohort with TB. The heterozygous genotype was significantly higher in patients with pulmonary TB and pulmonary and extrapulmonary TB than in patients with LTBI.

A meta-analysis has also reported TLR1, TLR2 and TLR6 polymorphisms associated with pulmonary tuberculosis in different geographical regions. The G1805T polymorphism in TLR1 was shown to increase susceptibility to TB in Africans and American Hispanic subjects, the G2258A polymorphism in TLR2 was associated with increased TB risk in Asians and Europeans and the C745T polymorphism in TLR6 was associated with decreased TB risk (Zhang *et al.* 2013).

1.8.2 Natural resistance-associated macrophage protein (NRAMP)

Studies of an inbred strain of mice with increased susceptibility to mycobacteria, salmonella and leishmania infections led to the identification of the Nramp1 gene located on chromosome 1, designated Bcg, Ity or Lsh (Bellamy and Hill 1998, Bellamy 2003, Plant and Glynn 1979, Crocker *et al.* 1984, Blackwell 1989).

Gruenheld *et al.* in 1994, isolated the mouse Nramp2 gene and it was mapped to chromosome 15.

The human homologue of Nramp1, designated NRAMP1 or SLC11A1 (solute carrier family 11, member 1) was mapped to chromosome 2q35 (Cellier *et al.* 1994).

In 1995, Vidal *et al.* reported the isolation and characterization of the human NRAMP2 (SLC11A2) gene, localized on chromosome 12q13.

Liu *et al.* in 1995 identified nine sequence variants in NRAMP1 and linked two highly polymorphic microsatellite markers to NRAMP1.

After a study done on a Brazilian population, Shaw *et al.* 1997 concluded that the NRAMP1 gene is not a major gene involved in susceptibility to TB in these subjects. However, a number of other studies completed on different ethnic groups have shown an association of polymorphisms in the NRAMP1 gene with susceptibility to TB.

Bellamy *et al.* 1998 reported on the variations in the NRAMP1 gene and susceptibility to TB in West Africans. An association was found with four polymorphisms, a microsatellite polymorphism 5'(CA)_n, a SNP in intron 4 (469+14G/C) reported as INT4, D543N and a deletion in the 3' untranslated region 1729+55del4.

In a study done on aboriginal Canadians, an association between susceptibility to TB and NRAMP intragenic variants was found (Greenwood *et al.* 2000).

A study on a Japanese population has reported on NRAMP1 polymorphisms, clinical features of TB and susceptibility to disease (Abe *et al.* 2003). While no association was observed between

susceptibility and human NRAMP1 polymorphisms, the genetic variation in this gene may be associated with cavitation in patients with TB.

The 5' allele [(GT)₉] in the promoter of SLC11A1 has been associated with higher expression and resistance to tuberculosis and the 120bp allele [(GT)₁₀] has been associated with lower promoter activity and susceptibility to TB (Awomoyi *et al.* 2002, Hoal *et al.* 2004).

A more recent review and meta-analysis done by Li *et al.* 2011 looking at widely studied polymorphisms for 3' UTR, D543N, INT4 and 5' (GT)_n, reported that NRAMP1 may play an important role in host defence against development of TB.

These varied reports indicate that NRAMP is involved in susceptibility to mycobacterial infections in humans, but the effect is too weak to be the sole gene involved.

1.8.3 Interleukin 1 β and interleukin 1 receptor agonist (IL-1 β and IL-Ra)

The genes encoding the proinflammatory cytokine interleukin 1 β (IL-1 β) and its antagonist receptor interleukin 1 Ra (IL-Ra)(IL1 receptor agonist) are both located on chromosome 2.

They are strongly induced by *M. tb* and are secreted by monocytes, macrophages and dendritic cells. IL-1 β is expressed in excess at the site of disease in patients with TB (Law *et al.* 1996). A study done on Gujarati Asians in west London showed that a proinflammatory haplotype of IL-1 β and IL-1Ra was significantly more common in patients with TB pleurisy than in those with other types of TB or healthy *M. tb* sensitized healthy controls (Wilkinson *et al.* 1999).

1.8.4 Vitamin D and Vitamin D receptor

During the 19th century, cod liver oil and sunlight were widely used to treat patients with TB. Today, epidemiological evidence links vitamin D deficiency and TB.

Vitamin D is synthesized in the skin during exposure to sunlight and is metabolized by the liver to 25-hydroxyvitamin D [25(OH)D], the major circulating vitamin D metabolite and standard measure of vitamin D status. 25(OH)D undergoes further hydroxylation to 1 alpha, 25 dihydroxy vitamin D₃ [1,25(OH)₂D₃], the hormonal form of vitamin D and immunomodulatory metabolite that enhances antimycobacterial activity (Liu *et al.* 2006, Martineau *et al.* 2007).

Early studies had shown that *in vitro* 1,25(OH)₂D₃ enhances the ability of human monocytes to restrict growth of *M. tb* (Rook *et al.* 1986) and that vitamin D exerts its effect via the Vitamin D receptor (VDR) which is present activated lymphocytes (Reichel *et al.* 1987).

In a study done by Martineau *et al.* in 2007, it was shown that 1,25(OH)₂D₃ reduced transcription and secretion of IFN γ , IL12p40 and TNF α in PBMCs and macrophages infected with mycobacteria (BCG and H37RV). Results showed that 1,25(OH)₂D₃ also suppressed BCG and *M. tb* in culture.

VDR polymorphisms were typed in the Gambian case-control study, previously discussed in section 1.9.2, in which NRAMP1 had been found to be associated with TB (Bellamy *et al.* 1998). Genetic variation in bone mineral density has been shown to be associated with SNPs in the VDR gene. These include an exon 9, codon 352, synonymous change, ATT to ATC creating a *Taq1*, restriction site (Morrison *et al.* 1994). Alleles were designated t and T, the presence

or absence of the restriction site denoted by t or T respectively. Analyzing the respective genotypes TT, Tt and tt, results suggested that tt homozygotes may be resistant to clinical TB, as subjects with the codon 352 tt genotype were significantly underrepresented among TB patients (Bellamy *et al.* 1999).

The prevalence of both vitamin D deficiency and TB is high among Asian immigrants in the United Kingdom (Bellamy 2003). A report by Martineau *et al.* 2007 showed by using the BCG lux assay, that a single dose of vitamin D enhances immunity to mycobacteria

Wilkinson *et al.* 2000 investigated the interaction between serum vitamin D (25-hydroxycholecalciferol) concentrations and VDR genotype with respect to susceptibility to TB. They typed three VDR polymorphisms defined by the presence of restriction endonuclease sites for *Taq1*, *Bsm1* and *Fok1*. There was no significant association between VDR genotype and TB, but the combination of codon 352 genotype TT/Tt and 25-hydroxycholecalciferol was associated with disease and the genotype ff or undetectable 25-hydroxycholecalciferol was strongly associated with disease. The tt genotype was also found to be less frequent among Gujarati Asian patients affected with TB in west London, compared to controls. In the Wilkinson study, the f allele containing the *Fok1* site was found to be associated with extrapulmonary disease.

Gao *et al.* 2010 did a meta-analysis on associations of the *FokI*, *TaqI*, *ApaI* and *BsmI* VDR polymorphisms with risk of TB infection and results supported the hypothesis that Vitamin D deficiency may play a role as risk factor during development of clinical disease.

Rathored *et al.* 2012 did a study in northern India, enrolling 897 participants as three groups (354 MDR-TB, 338 DS-PTB, 205 controls 205). Genotypic and allelic frequencies of *FokI*, *BsmI* and *TaqI* VDR polymorphisms and concentrations of 25(OH)D were analyzed. The *FokI* Ff genotype and *TaqI* t allele correlated positively with MDR-TB. The ff genotype and f allele of *FokI* frequency were higher in MDR-TB and drug sensitive TB groups. *BsmI* Bb genotype correlated inversely with MDR-TB. Serum 25(OD)D concentrations were significantly lowest in MDR-TB, correlating inversely with time to sputum smear conversion. This study concluded that VDR gene polymorphisms and hypovitaminosis D may predispose to MDR-TB and lower serum 25(OD)D may increase time to MDR-TB sputum negativity.

1.8.5 Vitamin D binding protein

The polymorphic system known as the group specific component (GC) was identified as the vitamin-binding protein (DBP) of human plasma.

Vitamin D metabolites in the circulation are bound to vitamin D binding protein (DBP). Double-band variants were called GC1 and single-band variants were named GC2 according to nomenclature adopted in 1978. (Cleve and Constans 1988). Two common polymorphisms at codons 416 (GAT to GAG, Asp to Glu) and 420 (ACG to AAG, Thr to Lys) of exon 11 of this gene give rise to three major electrophoretic variants, Gc1F (fast), Gc1S(slow) and Gc2. These Gc variants of the DBP differ in affinity for vitamin D metabolites that modulate antimycobacterial immunity (Martineau *et al.* 2010).

In a study using TB patients and healthy controls from Brazil, South Africa and Gujarati Asians in the UK, it was shown that an association between Gc genotype and susceptibility to TB is dependent on vitamin D status. The Gc2/2 genotype was strongly associated with

susceptibility to active disease in Gujarati Asians. This association was preserved if serum 25(OH)D was <20 nmol/l, but not if it was higher. No association was observed in the other ethnic groups (Martineau *et al.* 2010).

1.8.6 NOS2A

The nitric oxide synthase 2A gene (NOS2A) is induced in response to infections and cytokines, generating nitric oxide (Kun *et al.* 2001). Studies have shown NOS2A to be associated with TB.

Gomez *et al.* 2007 evaluated the influence of two NOS2A SNPs on the risk of developing pulmonary TB in a Northwestern Colombian population and found influence with one SNP.

In another study, a mixed case control association study of individuals with TB, relatives or close contacts was carried out. Ten NOS2A SNPs in African-Americans were associated with TB, three SNPs interacted with a SNP in TLR4 and five others interacted with a SNP in IFN γ R1, showing that variants may contribute to TB susceptibility and act synergistically with SNPs in other genes (Velez *et al.* 2009).

In a South African coloured population, association was found with NOS2A haplotypes consisting of two promoter polymorphisms reported (Moller *et al.* 2009).

1.8.7 Immunity related GTPase M (IRGM)

IRGM has been shown to be critical in regulatory autophagy as a means of disposing cystolic cellular structures and reducing the growth of intracellular pathogens, including *M. tb*. IRGM gene variants were studied in 2010 patients with pulmonary TB and 2346 unaffected

controls. The IRGM genotype -261TT was negatively associated with *M.tb* disease (Intemann *et al.* 2009).

1.9 Additional Association Studies

Today, microarray technology is used for identification of RNA expression signatures. Some reported studies are tabulated in table 1.8.

Study findings	Country	Reference
Whole blood RNA expression: distinct gene expression patterns identified in patients with active, recurrent, latent and cured TB (South African adults)	South Africa	Mistry <i>et al.</i> 2007
PBMC RNA expression: differential gene expression patterns identified in TB patients and healthy controls (German adults)	Germany	Jacobsen <i>et al.</i> 2007
Whole blood RNA expression: 393 gene transcript identified, differentiating between active TB, latent TB and healthy controls; 86 gene transcript identified, differentiating active TB from other inflammatory conditions and infectious diseases (South African and British adults)	Multiple	Berry <i>et al.</i> 2010
Whole blood RNA expression: 127 probeset representing 77 genes differentiated between active TB, latent TB, BCG vaccinated individuals and healthy controls (Brazilian adults)	Brazil	Lesho <i>et al.</i> 2011
PBMC RNA expression: 506 differentially expressed genes identified, distinguished between active TB, latent TB and healthy controls (Chinese adults)	China	Lu <i>et al.</i> 2011
PBMC RNA expression: 17 microRNAs differentially expressed, distinguished between active TB, latent TB and healthy controls (Chinese adults)	China	Wang <i>et al.</i> 2011
Whole blood RNA expression: different gene expression patterns identified in individuals with active TB and sarcoidosis (German adults)	Germany	Maertzdorf <i>et al.</i> 2012
Whole blood RNA expression: innate immune type 1 interferon signaling cascade may be used for monitoring active TB disease (Indonesian adults)	Netherlands	Ottenhof <i>et al.</i> 2012
Whole blood RNA expression: 116 gene expression pattern identified, distinguishing between active TB, latent TB and healthy controls (Warao Amerindian children)	Netherlands	Verhagen <i>et al.</i> 2012

Table 1.8: Reported RNA expression signatures associated with TB

Several GWAS have been carried out on different cohorts in several countries, identifying chromosomal regions associated with TB (Moller *et al.* 2012).

These are tabulated in table 1.9.

Genetic region	Country	Reference
15q11-13	South Africa	Bellamy <i>et al.</i> 2000
Xg	The Gambia	Bellamy <i>et al.</i> 2000
10q26-13, 11q12.3, 20p12.1	Brazil	Miller <i>et al.</i> 2004
8q12-q13	Morocco	Baghdadi <i>et al.</i> 2006
6p21-q23	South Africa	Cooke <i>et al.</i> 2008
20q13.31-33	Malawi	Cooke <i>et al.</i> 2008
2q21-2q24, 5p13-5q22, 7p22-7p21, 29q13	Uganda	Stein <i>et al.</i> 2008
18q11.2	Ghana, The Gambia, Malawi	Thye <i>et al.</i> 2010, Thye <i>et al.</i> 2012
5q23.2-31.3, 17p13.3-13.1, 20p13-12.3	Thailand	Mahasirimongkol <i>et al.</i> 2012
JAG1, DYNLRB2, EBF1, TMEFF2, CCL17, HAUS6, PENK, TXNDC4	Indonesia	Png <i>et al.</i> 2012

Table 1.9: Reported GWAS associated with TB

These GWAS have added to the understanding of the molecular mechanisms of TB disease. However, many of the genetic associations

reported by the early candidate gene studies cannot be replicated by GWAS Qu *et al.* 2013.

This emphasizes the importance of both types of investigations.

Advancement of technology, genomic capture, including exome capture, is allowing for investigation of complete coding regions of the genome (Teer and Mullikin 2010).

As genetic regions shown to be important for immunity to TB are identified, relevant genes are being targeted.

A functional promoter polymorphism has now been identified in the *IFITM3* gene on chromosome 11p15, a susceptibility locus for tuberculosis in a Han Chinese population. *IFITM3* encodes for interferon inducible transmembrane protein 3, acting as an effector molecule for IFN γ . A case control study has shown a significant association between the rs3888188 polymorphism and susceptibility to TB. The rs3888188 G allele was found to be more frequent in TB patients and functional assays showed reduced promoter activity.

The genetic effect of TB susceptibility is further complicated by confounding effects of environmental factors and exposure to TB.

1.10 Concluding statement

In this chapter I have provided an overview of selected known genetic factors within and outside of the IL12/IL23/IFN γ pathway that are associated with mycobacterial diseases in general and *M. tb* disease more specifically.

1.11 This study

The work done for this thesis was part of a large Burroughs-Wellcome funded collaborative study that started in 2000 to look at the immunogenetics of susceptibility to mycobacterial infection. At that time, the greatest evidence to support an underlying genetic basis for TB had come from studies on patients with single gene defects which showed that up-regulation of the macrophage through the IL12/IL23/IFN γ pathway was a fundamental step in the containment of infection with mycobacteria. The majority of subjects with MSMD are susceptible to infections with NTM, but a small subset of this group, have been reported to be susceptible exclusively to *M. tb*. Observations from patients with MSMD also raise the possibility that a substantial proportion of children with disseminated TB have a Mendelian predisposition to disease.

The IL12p40 promoter had not been extensively screened and was a potential candidate gene for study. The IL12p40 promoter and the IFN γ genes were thus chosen for genetic screening in this study.

1.11.1 Hypothesis

Susceptibility to primary progressive TB of childhood may result from polymorphisms and mutations in genes within the IL12/IL23/IFN γ pathway. IL12p40 promoter genotype may be predictive of an immune phenotype that determines susceptibility to TB disease.

1.11.2 Aims

The aim of the work for this thesis was to investigate the genetic variation of the IL12P40 promoter for association with TB disease.

1.11.3 Objectives

1. To screen selected paediatric and adult cohorts in the Western Cape, South Africa, for DNA changes in the IL12p40 promoter.
2. To evaluate the functional relevance of IL12p40 promoter SNPs identified by a luciferase reporter assay.
3. To investigate the immunological phenotype in the paediatric cohort of TB cases screened by measurement of secreted cytokines, $\text{TNF}\alpha$, IL12, $\text{IFN}\gamma$ and the cytokine receptor IL12R β 1.

CHAPTER 2: The IL12 p40 gene promoter

2.1 Introduction

As IL12 is an important component of the IL12/IL23/IFN γ pathway, it was postulated that any mutations in the IL12p40 gene promoter would have the potential to be of clinical relevance. Promoter sites are important for gene function and mutations in these regions can be beneficial or detrimental.

The major focus of the genetic component of the work for my thesis was the screening of the IL12p40 gene promoter for DNA changes.

In this chapter I review the human IL12p40 gene promoter.

In 2004 there was a comprehensive screening of promoters in 1% of the human genome that was chosen for intensive study analysis in the first phase of the ENCODE (ENCyclopaedia Of DNA Elements) project (Encode Project Consortium 2004). The availability of genomic sequences of many eukaryotes has enabled the development of methods designed to analyse gene boundaries.

Various technologies have been used for inferring transcription start sites (TSS)s, including methods based on sequencing of cDNAs and those that use hybridization of RNA or cDNA to DNA probes. Some of the methods allow for targeting to a specific genomic region or gene and others are applied to whole genomes (Sandelin *et al.* 2007).

The integration of TSS, transcription factor binding data and phylogenetic information from multiple mammalian genomic sequences has the potential to identify new classes of genomic regulatory elements currently regarded as intergenic and it is likely that more promoter motifs are yet to be discovered.

2.1.1 Promoter types

The promoters of genes may be focused or dispersed and are comprised of the initiation site for the start of transcription and gene sequences upstream from the initiation site.

2.1.1.1 Core promoters

Core promoters which encompass the minimal portion of promoter sequence required to initiate transcription may exhibit combined properties of focused and dispersed promoters, having multiple dispersed start sites with one particularly strong start site (Juven-Gershon and Kadonaga 2010). Genome wide detection of TSSs has revealed that RNA polymerase II transcription initiates at millions of positions in mammalian genomes. Most core promoters do not have a single TSS but an array of closely located TSSs with different rates of initiation. Frith *et al.* 2008 showed that initiation events are clustered on the chromosomes. They showed that the TSSs can be predicted with high accuracy by sequence.

Studies have demonstrated that core promoters are diverse and complex and that specific DNA sequence elements and patterns are associated with them. These elements are not universal, each is present in only a subset of core promoters and some core promoters appear to lack all the known core promoter elements. These patterns have important characteristics that are linked to the expression of downstream genes. Different elements can co-occur in the same promoter, but some combinations are more likely than others.

2.1.1.2 Focused promoters

Focused promoters, also known as a single peak promoters or sharp promoters contain either a single TSS or a cluster of start sites over a

narrow region of several nucleotides and are usually associated with regulated genes (Juven-Gershon *et al.* 2008, Juven-Gershon and Kadonaga 2010). Most eukaryotic core promoters appear to be focused promoters, but in vertebrates, only about a third or less are focused promoters, present in a small group of biologically important genes. Focused promoter properties in vertebrates are dependent on the presence or absence of sequence motifs which serve as binding sites for transcription factors (Juven-Gershon and Kadonaga 2010). The analysis of focused core promoters led to the discovery of sequence motifs such as the TATAbox, BRE^u (upstream transcription initiator factor IIB [TBIIB] recognition element), BRE_d (downstream TBIIB recognition element), Inr (initiator), MTE (motif ten element), DPE (downstream promoter element), DCE (downstream core element), and XCPEI (X core promoter element I) (Sandelin *et al.* 2007, Juven-Gershon and Kadonaga 2010). The focused core promoter typically encompasses -40 to +40 nucleotides, which span relative to the +1 TSS and is the location at which the RNA polymerase II machinery initiates transcription (Juven-Gershon and Kadonaga 2010).

2.1.1.3 Dispersed promoters

Dispersed promoters are also known as broad promoters. In dispersed initiation there are multiple weak start sites over a broad region of about 50 – 100 nucleotides and this type of promoter is usually present in constitutive genes. In vertebrates, 70% of genes have dispersed promoters which are typically found in CpG islands (Juven-Gershon and Kadonaga 2010). Dispersed promoters generally lack BRE, TATA, DPE and MTE sequence motifs (Sandelin *et al.* 2007).

2.1.1.4 Bidirectional promoters

Genome wide analysis has also shown that there are genes with bidirectional promoters that are usually broad, with TSSs on opposite strands of DNA separated by less than 1kb, (Trinkelein *et al.* 2004).

2.1.1.5 Alternative exonic promoters

Some genes have more than one promoter region used in different contexts or tissues or to produce different protein products. These alternative promoters contribute to the complexity of the human proteome (Carninci *et al.* 2006). Weak alternative exonic promoters have also been identified. These exonic TSSs tend to be more prevalent in tissue specific genes (Carninci *et al.* 2006).

2.2 Reported promoter mutations

Promoter mutations have been described in many genes and may have major, subtle or negligible effects on gene expression.

Promoter mutations having clinical relevance have been reported.

An example of a promoter mutation having a major, beneficial effect is the C-46T mutation in the gene encoding the Duffy blood group antigen, the erythrocyte receptor for *Plasmodium vivax* malaria parasite, lying within a GATA motif. The mutation disrupts the binding site of the GATA1 erythroid transcription factor, impairing promoter activity. This causes resistance to infection and disease (Tournamille *et al.* 1995).

Several beneficial mutations have been identified in the IFN γ R1 gene promoter, an important component of the IL12/IL23/IFN γ pathway.

A paediatric case control study in the Mandinka ethnic group in The Gambia, looking at four promoter polymorphisms at +95, -56, -270 and -470 in the IFN γ R1 gene, showed that subjects heterozygous for the C-56T mutation appeared to be protected against cerebral malaria. The -470delTT was associated with protection against severe malaria in general. The subjects with these polymorphisms all had an additional A-611G polymorphism (Koch *et al.* 2002).

An adult study showed no association between promoter mutations at +95, -56, -270, -470, -611 and pulmonary TB in a Gambian cohort (Awomoyi *et al.* 2004).

Another study tested functionality of IFN γ R1 promoter mutations at -56, -72, -169, -255, -470 and -611 in African American, Caucasian and Korean subjects but found no association with mycobacterial disease, including pulmonary tuberculosis and NTM infections (Rosenzweig *et al.* 2004).

In the work for her thesis, Dr Suzanne Anderson identified five SNPs, -470/471 TTdel, C-255T, T-181G, T-72C, C-56T in the IFN γ R1 gene promoter in the cohort of cases and controls screened. The T-181G mutation was novel. The relatively small size of the cohorts studied, resulted in insufficient power to detect any effect on TB susceptibility (Anderson 2007).

2.3 The IL12p40 gene promoter

I will now describe the structure of the IL12p40 promoter, from its initial description in murine studies to more recent work on the human promoter.

2.3.1 Murine studies

In 1995, Murphy *et al.* defined the genomic organization and promoter sequence of the murine IL12p40 promoter, working with a 4.3kb proximal promoter region consisting of proximal 5' sequence, 1st exon and 1st intron. The aim of the study was to understand IL12 induction by bacterial products and IFN γ and they demonstrated several important features of the IL12p40 gene regulation in macrophages. They focused on the regulation of the IL12p40 subunit because it is induced by bacterial stimulation whereas the IL12p35 is constitutive.

By stimulating murine cells from peritoneal exudates with LPS, in the absence and presence of IFN γ , they measured IL12 production by ELISA and demonstrated that IFN γ caused a twenty-fold increase in the level of IL12 produced by LPS alone. Based on these results, the J774 murine macrophage cell line was used for transfection experiments with luciferase based murine IL12p40 promoter constructs. Cells transfected with constructs containing 4.3kb of proximal 5' sequence, first exon and first intron, showed LPS inducible and IFN γ regulated luciferase activity, with 13-fold increase above background with LPS and 21-fold above background with addition of IFN γ . Elements were determined using deletion mutants, restriction mapping, hybridization to IL12p40 specific oligonucleotides, PCR and sequencing.

To map the elements, various luciferase based deletion mutant reporter constructs from 40 to 4300bp of the 5' promoter sequence were prepared and the activity examined. No loss of promoter activity was seen from -703 to the TSS, but a deletion of 7bp between -132 and -125 markedly reduced the activity, suggesting the location of a *cis* acting element, which regulates expression of a gene located on

the same molecule of DNA, usually upstream of the coding sequence it controls.

These results suggested that the murine IL12p40 promoter sequence between -132 and -125 conferred LPS inducibility and IFN γ augmentation.

To identify factors contributing to inducible activity, overlapping oligonucleotide probes were used. The ⁻¹³¹AAAATTCCCC⁻¹²¹ sequence from -131 to -121 had 8 of 10 nucleotides that make up the identity of an NFkB consensus sequence.

This suggested the presence of a potential half site as a target for NFkB binding in which two adenine residues (-131 to -130) replace two conserved guanine residues common to reported functional NFkB sites (Baeuerle and Henkel 1994).

Using electrophoretic mobility shift assays (EMSA) and anti-sera to Rel transcription factors, this group localized a novel sequence that bound the rel family of transcription factors. They also localized inducible activity of the promoter to the sequence GGGAATTTA from -132 to -122. To test the functional role of the NFkB site for IL12p40 promoter activity, luciferase constructs with 2bp mutations were prepared. The native sequence conferred 15-fold inducibility without IFN γ pretreatment and 28-fold LPS inducibility with IFN γ pretreatment. Altering the ⁻¹³¹AA⁻¹³⁰ to a ⁻¹³¹GG⁻¹³⁰ mutation, converting the sequence to a full NFkB site, markedly increased the promoter activity.

NFkB mediates the induction of several proinflammatory molecules. Previous studies have described authentic NFkB binding sites with mismatches at one of these terminal residues, but this study was the first to show a site with both residues as mismatches.

A consensus sequence GAGGAA from -143 to -138 for B cell and macrophage specific transcription factor PU.1 lies immediately upstream of the NFkB site. After conducting EMSA experiments and comparing activities of IL12p40 luciferase reporter constructs which differed in regions containing PU.1 consensus sequences, they concluded that this site contributed to the activity of the murine IL12p40 promoter, but was not critical for induced promoter activity.

The work done by this group was novel in that they identified functional promoter regions that confer LPS inducibility and IFN γ augmentation and that transcription of IL12p40 was shown to be induced via a NFkB half site.

2.3.2 Human studies

IL12p40 gene activation is the result of a complex interplay between multiple inducible transcription factors. A number of detailed studies on isolated PBMC and macrophage cell lines have teased out this interplay and are described in the this section.

Early studies of the human IL12p40 gene promoter, identified important binding factors and repressor elements (Ma *et al.* 1996, Ma *et al.* 1997, Gri *et al.* 1998, Becker *et al.* 2001).

Ma *et al.* in 1997 did additional work, showing the comparison of genetic organization of the human and murine IL12p40 gene promoters. Several putative sequence motifs are highly conserved between the two IL12p40 promoters, suggesting their functional significance. Both promoters show a fairly high degree of homology up to approximately -400 with respect to the TSS. After this, the homology breaks down with large gaps in between the homologous regions.

In 1996 Ma *et al.* did experiments showing that IFN γ enhances IL12 production by priming phagocytic cells for LPS induced transcription of the IL12p40 gene. They studied the production and quantitation of IL12 by radio immunoassay (RIA) and ELISA after stimulation of PBMCs with IFN γ and LPS. In PBMCs, IFN γ alone did not induce significant IL12p40 production, LPS alone did induce a five-fold increase over unstimulated cells and IFN γ pretreatment resulted in a 75- fold stimulation of IL12p40. Cells treated simultaneously with IFN γ and LPS, produced marginally increased IL12p40 production compared to those pretreated with IFN γ , followed by LPS.

This group also did experiments with a 3.3kb human IL12p40 promoter reporter construct from +56 to the 5' end of the 3.3 kb. This was linked to a fruit fly luciferase reporter construct pXP2 at the *Pst*I site which was used for transient transfections in a number of myeloid and lymphocytic cell lines. Transfection was done by electroporation.

The cloned promoter construct was shown to be silent in two human T cell lines, constitutively active in two human EBV+ B lymphoblastoid cell lines and LPS-inducible in the human THP-1 and mouse RAW264.7 monocytic cell lines. As the RAW264.7 cell line is easily transfectable and regulates the endogenous IL12p40 gene in response to IFN γ and LPS similarly to that in human monocytes, it was used for analysis of regulation of the cloned human promoter.

Luciferase activity was measured and values normalized against the internal control β galactosidase, to correct for transfection efficiency. Effects of folds increase induced by IFN γ and LPS were replicated by these luciferase assays.

This showed that the cloned 3.3kb fragment of the IL12p40 promoter could mimic the behaviour of the endogenous IL12p40 promoter in transient transfection assays in terms of cell type specificity and responsiveness to IFN γ and LPS.

Deletion mutant constructs generated by PCR were tested for their ability to induce transcription of the reporter-luciferase gene in response to IFN γ and LPS. Promoter regions truncated at -222 showed that 50% reduction in expression when compared to the 3.3kb sections. This indicated that upstream elements enhanced transcription. A more severely truncated region at residue -204 showed a substantial reduction of inducibility by IFN γ and LPS. A potential motif TTTCCT was located between -204 and -222. A GGAA motif has been established as the consensus motif for the ets family of transcription factors. A 5bp deletion of this sequence resulted in complete unresponsiveness to IFN γ and LPS. Additional experiments with an ets-2 expression vector indicated that ets-2 was mediated by this element. The construct truncated at -222 was active in monocytic cells and B cells, but silent when transfected into T cell lines, indicating regulation in a tissue specific manner as was observed in the 3.3kb promoter. The construct truncated at -204 showed low, but consistent, inducibility by IFN γ and LPS, indicating that a downstream element may be responsible for this persistent inducibility. It was thought that the NFkB site was responsible for this, as it bound to a consensus site CCCTTAAAGT located at -116 to -107 in response to LPS.

Two other elements, interferon regulatory factor 1 (IRF-1), important for gene induction, located at -730 to -719 and nuclear factor for IL6 (NF-IL6), a transcription factor, at -520 to -512 were studied by

deletion analysis. Experiments showed that these elements did not result in decreased response to IFN γ and LPS stimulation.

In a further study, Ma *et al.* 1997 showed that within the -400 proximal promoter region, several putative transcription factor binding sites, the ets-2, PU.1 and NFkB-like elements were very well conserved and that the ets-2 element at -211 to -207 was a functionally critical site. It reacted with nuclear complex termed F1 which was highly inducible by IFN γ treatment for 16 hours or LPS stimulation for 8 hours.

Ets-2 and a novel, antigenically related protein with approximate molecular mass of 109kDa, plus additional components including IRF1 and cRel were shown to be part of the F1 complex. This novel protein was designated GL109, a nuclear factor inducible by IFN γ and LPS. It is part of a complex including IRF1 and possibly NFkB c-Rel that interacts with the ets element in the IL12p40 promoter. The NFkB and ets sites appeared to be equally critical for inducible promoter activity in macrophage cell lines, the NFkB having a more dominant role in constitutive IL12p40 promoter activity in EBV+ B cells. Ets-2 and c-Rel are transiently expressed in B, T and monocytic cell lines, synergistically activated with the IL12p40 promoter, indicating a lack of requirement for cell type or stimulant specific transcription factors.

In 1998, Gri *et al.* also worked with the 3.3kb human IL12p40 promoter reporter construct, from +108 to -3300, using various cell lines including human EBV transformed B lymphoblastoid cell line, RPMI-8866, EBV-Burkitt's lymphoma cell line BJAB, T cell line Jurkat and the murine macrophage cell line RAW 264.7. The promoter fragment was cloned into a luciferase reporter construct pXP2 at the *Pst*I site and transfection was done by electroporation, using BJAB

cells, Jurkat cells, RPMI 8866 cells and RAW 264.7 cells. Luciferase activity was corrected for transfection efficiency by normalizing to the measured CMV β galactosidase internal control level. Activity was expressed as fold induction above the background of cells transfected with the promoterless plasmid. Luciferase activity of the +108 to -3300 construct and a series of nested 5' deletion constructs was measured. Luciferase activity of the full construct was 65-fold that of the promoterless vector and measurement of the deletion mutant constructs in EBV+BCL suggests that the region between -222 and -28 is most crucial for constitutive promoter activity on the BJAB (EBV+BCL) cells. Two essential *cis* elements, an NFkB site and an ets site have been localized within the promoter. By preparing a series of promoters with defined mutations, the functional roles of the NFkB and ets sites at -117 and -212 was evaluated. Analysis of the activity of the constructs in transiently transfected EBV+BCL showed a marked increase in the luciferase activity of promoter constructs mutated at the NFkB site, but only a 50% decrease for the ets-2 deletion mutants. In contrast, analysis of constructs in the RAW 264.7 cells showed that mutations at both these sites almost completely abolished the inducibility of the IL12p40 promoter by $\text{INF}\gamma$ and LPS. These results suggest that B cells use the same *cis* elements and transcription factors as macrophages do, but the relative role of each may vary. Using EMSA experiments, the NFkB site was shown to interact with p50 and c-Rel, members of the NFkB family and the ets site constitutively bound an ets-2 containing complex. This showed that full activation of the human IL12p40 promoter is a result of interplay between NFkB and Ets family members.

In 2001 Becker *et al.* also studied regulation of the human IL12p40 promoter construct from +66 to -747. This 800bp promoter fragment

was cloned into the luciferase pXP1 vector and RAW 264.7 cells transfected by the diethylaminoethyl (DEAE) method. The promoter was synergistically induced in RAW 264.7 cells upon stimulation with LPS plus $\text{IFN}\gamma$, showing a 9.5-fold induction compared with unstimulated cells.

In vivo footprinting in resting and activated human CD14⁺ monocytes was studied. Footprints for trans-activating proteins C/EBP, NF κ B, and ets were only found on stimulation with LPS and $\text{IFN}\gamma$. In contrast, footprinting over a purine rich sequence at -155, termed GA-12, a GATA sequence in the IL12 promoter, was observed in resting but not activated cells. Mutagenesis within the GA-12 sequence caused upregulation of inducible IL12p40 promoter, suggesting a repressor function at this site. The binding activity of the GA-12 binding protein GAP-12 was increased by IL4 and Prostaglandin 2 (PGE_2), inhibitors of IL12 expression, suggesting that GAP-12 binding may modulate IL12p40 gene expression.

These findings suggest that in unstimulated human monocytes, GAP-12 binds to the IL12p40 promoter, suppressing gene expression. Upon stimulation, GAP12 is displaced from the promoter and NF κ B p50/p65, C/EBP β and PU.1 promote the induction of IL12p40 gene expression.

These studies identified important binding and transcription factors in the IL12p40 gene promoter.

2.3.3 The human IL12p40 gene promoter

The genetic organization of the proximal region of the human IL12p40 gene promoter, a focused promoter with one TSS, is depicted in figure 2.1.

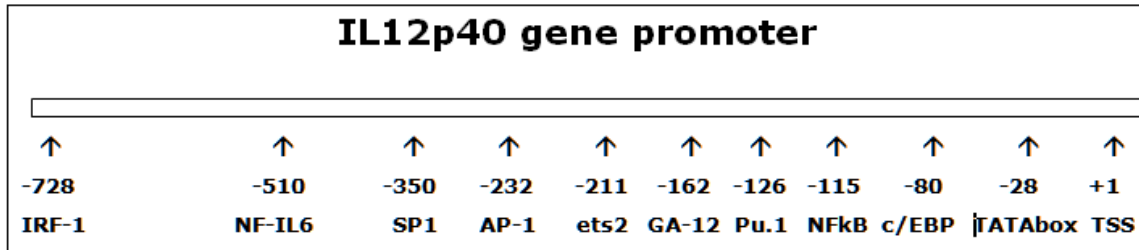


Figure 2.1: Schematic representation of the genetic organization of the proximal region of the human IL12p40 gene promoter showing TSS and transcription factors TATAbox, CCAAT/enhancer binding protein (c/EBP), nuclear factor kappa B (NFkB), PU.1, GA-12, epithelium specific transcription factor 2 (ets2), activator protein 1 (AP-1), specificity protein 1 (SP1), NF-IL6, interferon regulatory factor 1 (IRF-1).

Regulation of the IL12p40 gene is complex and involves various transcription factors and binding sites in the IL12p40 gene promoter. These are described in the following section.

2.3.3.1 TATAbox

The TATAbox, the most ancient and widely used core promoter motif throughout nature, was the first eukaryotic core promoter element to be identified (Michael L Goldberg PhD thesis Stanford University 1979, Smale and Kadonaga 2003). It has been conserved from archaea to humans (Reeve 2003) is also present in plants (Molina and Grotewold 2005, Yamamoto *et al.* 2007), but is only present in 10%-15% of mammalian core promoters (Carninci *et al.* 2006). The TATAbox assists in directing RNA polymerase II to the initiation site downstream on the DNA, ensuring that RNA polymerase II is correctly positioned.

The metazoan TATAbox consensus sequence is TATAWAAR where the upstream T is usually located at -31 or -30 relative to the A+1 (or G+1) position in the Inr (Carninci *et al.* 2006, Ponjavic *et al.* 2006).

TATAbox is recognized and bound by TATAbox binding protein (TBP) which is a subunit of transcription initiation factor IID (TFIID) complex in eukaryotes.

Statistical analysis (Ponjavic *et al.* 2006) and structural studies (Hahn 2004) show that the TATAbox position can vary by 28-34 bp from the first T in TATAA consensus to the major initiation peak with a distance of 30-31 bp being preferred.

As previously discussed, the presence of a TATAbox is associated with promoters that have a sharply defined TSS. However, not all sharp promoters have a TATAbox.

Ponjavic *et al.* 2006 showed that tissue specificity is strongest if the TATAbox is -30 bp or -31 bp relative to dominant TSS and that the initiation site consensus varies with the TATA-TSS distance. Studies show that the longer TATA-TSS distances (32-34 bp) are used more frequently than the shorter distances (28-29 bp) (Molina and Grotewald 2005, Ponjavic *et al.* 2006).

Precise transcription initiation in TATAbox containing promoters generally requires both the TATAbox and Inr like element (O'Shea-Greenfield and Smale 1992).

Most promoters regardless of class, lack the Inr consensus sequence but even in its absence, the precise start point of RNA polymerase II mediated transcription is not random, but specific.

2.3.3.2 CCAAT/enhancer binding protein (c/EBP)

Maehara *et al.* 1999 showed that NFkB and c/EBP act synergistically to enhance gene transcription induced by LPS and IFN γ (Maehara *et al.* 1999).

2.3.3.3 Nuclear factor kappa B (NFkB)

Nuclear factor kappa B (NFkB) dimers are ubiquitously expressed and their release into the nucleus initiates the process of gene transcription. This is discussed in chapter 1.

2.3.3.4 PU.1

Transcription factor PU.1 is tissue specific and expressed in cells of the haematopoietic lineage, including macrophages, B lymphocytes and granulocytes (Celada *et al.* 1996).

2.3.3.5 GA-12

GA-12 (GATA sequence in the IL12p40 promoter) is a repressor element of IL12p40. Saito *et al.* 2006 reported impairment of IL12p40 production by the mouse macrophage cell line RAW264.7 in response to LPS. Activation of GA-12 was detected, rather than suppression of promoter elements such as NFkB, AP-1 and IRF-1. This was accompanied by hyperactivation of the extracellular signal-related kinase (ERK). When the ERK activation was suppressed by the UO126 inhibitor, the IL12p40 production increased and GA-12 activation decreased.

2.3.3.6 Epithelium Specific Transcription factor 2 (ets2)

The first member of the ets (E twenty six) family of transcription factors was identified as a gene transduced by the leukaemia virus E26. All ets family members are identified by a highly conserved DNA

binding domain, the ets domain. Ets factors may act as repressors or activators of transcription. (Sharrocks 2001).

2.3.3.7 Activator Protein 1 (AP-1)

AP-1 is a heterodimeric protein made of proteins belonging to the c-Fos, c-Jun, ATF (activity transcription factor) and JDP (Jun dimerization protein) families. It regulates gene expression in response to a variety of stimuli, including normal development and neoplastic transformation, functioning within the complexity of many cell pathways (Hess *et al.* 2004).

2.3.3.8 Specificity Protein 1 (SP1)

SP1 is a human transcription factor, belonging to the Sp/KLF family of transcription factors and is involved in gene expression in the early development of an organism (Paonessa *et al.* 2013).

2.3.3.9 NF-IL6 (nuclear factor for IL6)

Identification of the NF-IL6 transcriptional regulator has been important in recognizing the importance of its potential role in multi drug resistant 1 (MDR1) gene induction (Combates *et al.* 1994). Single binding sites for NF-IL6 and NFkB are present in the promoter of the IFN γ gene and they act synergistically (Matsusaka *et al.* 1993).

2.3.3.10 Interferon-Regulatory Factor Transcription Factor 1 (IRF1)

The IRF family of transcription factors is part of the winged helix-turn-helix DNA-binding domain superfamily. Members of this family share a highly conserved 120 amino acid DNA-binding domain that recognizes ISRE consensus DNA sequences. The ISREs are found in promoters of type I IFNs and many genes including type I IFN-induced genes as

discussed in chapter 1, section 1.3.1.2. IRFS are involved in many immune processes.

The complex interplay of transcription factors and binding sites is illustrated in figure 2.2.

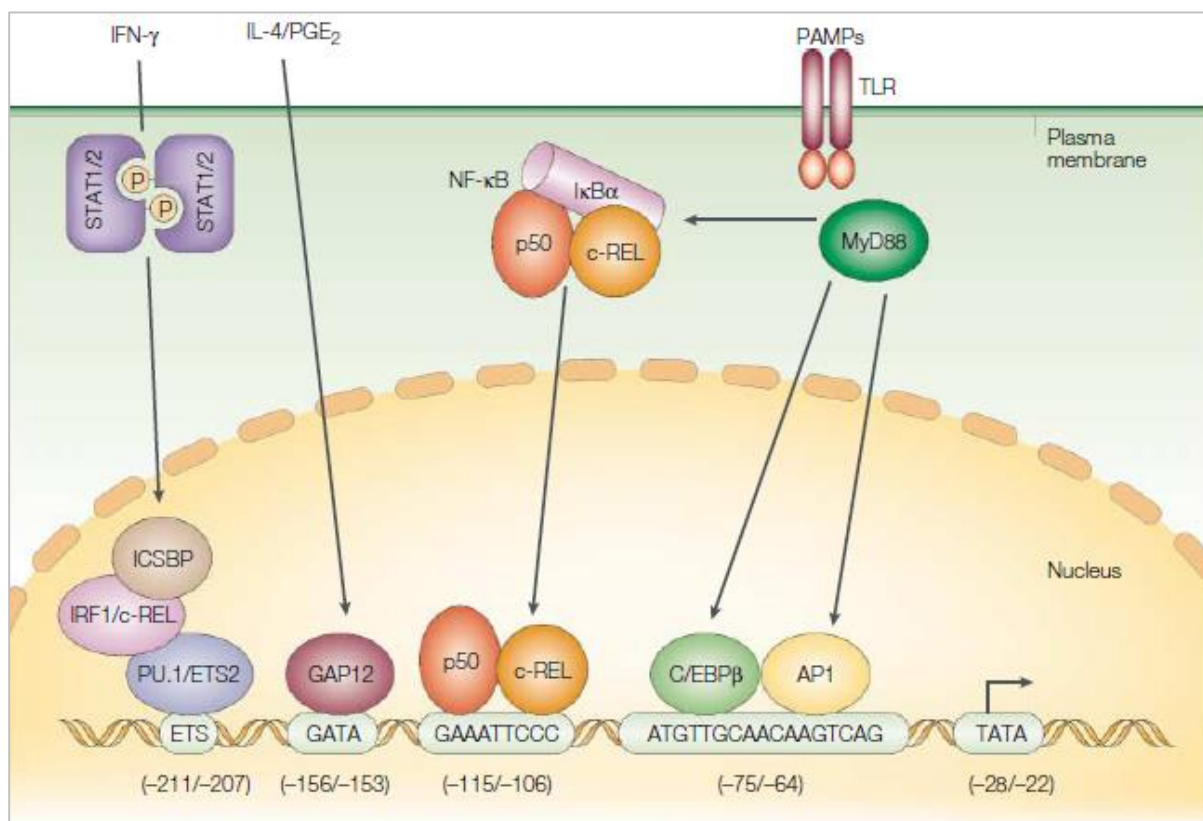


Figure 2.2: Transcriptional regulation of the gene encoding IL12p40.

Source: Trinchieri 2003

2.4 Reported mutations in the human IL12p40 promoter

There have been several publications reporting mutations in the IL12p40 (IL12B) promoter (Pravica *et al.* in 2000, Morahan *et al.* 2001, Morahan *et al.* 2002a, Morahan *et al.* 2002b, Khoo *et al.* 2004, Muller-Bergaus *et al.* 2004, Marquet *et al.* 2008, Morris *et al.* 2011).

Pravica *et al.* in 2000 reported a G to T change at position -1287 in a single individual in a study of adults in the UK, but no clinical data were given and therefore, it is not known if this had any functional effect.

There have been several publications about a microinsertion event (GC)del(CTCTAA)ins 3kb upstream of the transcription start site. The normal, shorter allele was notated IL12Bpro2 and the mutated allele IL12Bpro1.

Morahan *et al.* in 2002a studied the genetics of this microinsertion event in conjunction with a polymorphism A16974C in the IL12B 3' untranslated region (UTR). The A16974C polymorphism had been found to be associated with development of Type I Diabetes and expression of higher levels of IL12 *in vitro*. The A allele was notated IL12B 3'UTR1 and the C allele notated IL12B 3'UTR2 (Morahan *et al.* 2001). Examining the four haplotypes, the outcome of malaria was studied with respect to the various polymorphisms. An association was found between the microinsertion event genotype and cerebral malaria in Tanzanian children. IL12Bpro1 homozygosity was functionally associated with lower nitrous oxide production and therefore it was found that homozygosity for the IL12Bpro1/3'UTR2 haplotype conferred greater susceptibility to death from cerebral malaria in Tanzanian children, but not in Kenyan children.

Morahan *et al.* in 2002b also linked this microinsertion event with the severity of atopic and non atopic asthma in children in Australia. The children who were heterozygous for this polymorphism were at the greatest risk for progression to severe asthma. However, another study done by Khoo *et al.* in 2004 found no association with asthma or atopy and the microinsertion event in Australian children and adults.

Muller-Bergaus *et al.* in 2004 published work on the microinsertion event in a cohort of Australian adults linking it to the secretion of IL12. They observed that homozygotes for the longer IL12Bpro1 allele were unable to secrete IL12.

This microinsertion event was also found to correlate with secretion of IL12, having an impact on the clinical course of disease in patients with melanoma (Schadendorf *et al.* 2006).

A study done on a cohort of families in Mali by Marquet *et al.* in 2008 observed that the heterozygous genotype GC/CTCTAA was overtransmitted in children with cerebral malaria.

Morris *et al.* 2011 investigated whether polymorphisms in the IL12p40 promoter were associated with susceptibility to pulmonary TB in two West African populations, The Gambia and Guinea-Bissau and in two independent populations from North and South America. Nine polymorphisms were identified including a 3 bp insertion in the promoter, reported as position 158692783 with rs 17860508. No association was found with this promoter polymorphism.

A summary of these reported mutations in the human IL12p40 promoter are listed in table 2.1.

Mutation	Population	Age group	Associated condition studied	Reference
G-1287T	UK	Adults	Not reported	Pravicia <i>et al.</i> 2000
(GC)del(CTCTAA)ins and A16974C	Tanzania, Kenya	Children	Cerebral malaria	Morahan <i>et al.</i> 2002a
(GC)del(CTCTAA)ins	Australia	Children	Asthma	Morahan <i>et al.</i> 2002b
(GC)del(CTCTAA)ins	Australia	Children and adults	Asthma, atopy	Khoo <i>et al.</i> 2004
(GC)del(CTCTAA)ins	Australia	Adults	Secretion of IL12	Muller-Bergaus <i>et al.</i> 2004
(GC)del(CTCTAA)ins	Australia	Adults	Melanoma	Schadendorf <i>et al.</i> 2006
(GC)del(CTCTAA)ins	Mali	Children	Malaria	Marquet <i>et al.</i> 2008
3bp insertion	The Gambia, Guinea Bissau	Adults	Pulmonary tuberculosis	Morris <i>et al.</i> 2011

Table 2.1: Reported mutations in the human IL12p40 promoter

These studies report mutations in the IL12p40 promoter, many far upstream of the documented transcription and binding sites (Pravicia *et al.* 2000, Morahan *et al.* 2002a, Morahan 2002b, Khoo *et al.* 2004, Muller-Berghaus *et al.* 2004, Schadendorf *et al.* 2006, Marquet *et al.* 2008).

These studies also indicate that the heterozygous genotype is often functionally significant and that conducting studies on different ethnic

groups and populations is important, as variable effects can be observed between different populations.

In chapter 3, I describe the methodology used for promoter screening and frequencies of detected SNPs.

CHAPTER 3: IL12p40 promoter screening and SNP frequencies

3.1 Introduction and Ethics statement

This chapter describes the cohort screened, methodology and work carried out to identify sequence changes in the IL12p40 promoter.

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Research Ethics Committee, University of Cape Town (Reference number: 013/2000). All patients and controls provided written, informed consent for the collection of samples and subsequent analysis.

3.2 Study group

3.2.1 Paediatric cases

3.2.1.1 Recruitment of TB cases

A group of children of Xhosa origin and a group of children from the heterogeneous Coloured group were recruited to the study. The Coloureds are the predominant population in the Western Cape and are a mixed ethnic group of Malay origin mixed with either European or the indigenous Khoi and San people (Salmon and Martell 1994).

Recruitment of all TB cases was made mainly at Red Cross War Memorial Children's Hospital (RCWMCH) and local community clinics within two health districts of the City of Cape Town. These were firstly the predominantly Xhosa, formal urban settlements of Langa and Guguletu within the City of Cape Town and the informal Xhosa settlement of Khayelitsha. In addition, a smaller number of children

were recruited from Coloured settlements within Cape Town Central and the South Peninsula.

3.2.1.2 Method of recruitment

TB cases suitable for recruitment were identified by Dr Suzanne Anderson (SA) using three main sources of information:

- Past and current admissions to the RCWMCH and associated University of Cape Town Teaching Hospitals.
- Past and current TB culture results from the microbiology laboratory at the RCWMCH where, on average, 100-120 culture positive cases are identified annually.
- Official TB notification records from the City of Cape Town Health Department. The name, address, age and type of TB of each notified case is provided on their database. The majority of cases came from local community clinics and day hospitals and confirmatory microbiology was therefore only available in a minority of cases.

Children who had either active TB or a past history of TB disease based on hospital records were recruited to the study. Newly diagnosed active TB cases were recruited at the time of hospital admission and commencement of anti-TB chemotherapy, while past history cases were enrolled at least one year after initial diagnosis. The rationale for not recruiting past history cases until this time was to ensure that the *in vitro* functional studies of cytokine responses were unaffected by the acute disease process itself.

Exclusion criteria were: age over 14 years, HIV seropositivity, any other underlying immunodeficiency, children treated with

glucocorticosteroids or other immunosuppressive drugs for a prolonged time period.

3.2.1.3 Diagnostic classification

TB cases were classified as active or past history in the following categories:

- **Definite TB** - diagnosis confirmed by culture of *M. tb* from sputum, gastric aspirate, CSF, other body fluids or biopsy material.
- **Highly Probable TB** – acid fast bacilli identified in sputum, gastric aspirate, CSF, other body fluids or on histological examination of biopsy material.

- **Probable TB**

– adapted from World Health Organisation criteria (WHO 1983 Report No EPI/GEN/83/4. Provisional guidelines for the diagnosis and classification of the EPI target diseases for primary healthcare, surveillance and special studies) as

An ill child with a history of contact with suspected or confirmed case of pulmonary TB

plus any one of the following criteria:

- A history of contact with a confirmed adult pulmonary TB case.
- Suggestive changes of active TB on chest X-Ray.
- Loss of weight, or cough for 2 weeks, or no response to antibiotic treatment and/or a favourable response to anti TB treatment.

- Suggestive histological findings in biopsy material.
- A Mantoux response of $\geq 15\text{mm}^*$.

*Note, in this study the cut-off point for a positive Mantoux was set at 15mm which is well above the WHO recommended value of $>10\text{mm}$. This is in keeping with recruitment in a high TB prevalence environment (Donald 1999).

At the time of this study, classification of Probable TB was defined by this WHO 1983 report. Subsequently, the definition of Probable TB has become tighter and more specific (Graham *et al.* 2011).

- **Possible TB** - Symptoms of TB for more than two weeks (cough, fever, weight loss) and one of either:
 - a suggestive chest x-ray
 - a household contact
 - no alternative diagnosis established.
- Where an alternative diagnosis became apparent after recruitment, patients were re-categorised and excluded from further analysis in the study.
- **Past History of TB** – children were classified as past history cases if recruited 12 months or more after the initial diagnosis of TB and commencement of treatment.

A total of 252 paediatric TB cases were recruited comprising 90% Xhosa and 10% Coloured subjects. The clinical features and of paediatric cases and controls are summarized in tables 3.1 and 3.2.

	Active TB	Past History TB	Total
Definite	39	36	75
Highly Probable	6	3	9
Probable	90	18	108
Possible	33	27	60
Total	168	84	252

Table 3.1: Categories of paediatric TB cases

All active TB cases were seen and bled on three different occasions: at enrolment, six months into treatment and one year after enrolment. Clinical data were collected from all cases and blood collected for functional assays and DNA extraction on all enrolled subjects on every visit.

Past history cases were bled on two occasions, two to four weeks apart to check for stability of the functional phenotype.

Continuous Outcomes					
Cases			Controls		p value
	N	Median Age (IQR)	N	Median Age (IQR)	
All TB Cases	252	40 (20-87)	145	55 (30.5-91.5)	P=0.15**
PTB Cases	105	33(15-65)			
EPTB Cases	145	48(24-112)			
Categorical Outcomes					
	Cases		Controls		p value
	N	%	N	%	
Sex	252		145		p = 0.005**
Female	117	46	77	53	
Male	135	58	67	47	
BCG vaccination					
Yes	119	47	63	43	p = 0.91**
No	77	31	42	29	
Not recorded on RTHC*	56	22	40	28	
Mantoux					
0-14mm	41	10(0-11)	94	0(0-0)	p = 0.00**
≥15mm	94	20(16-22)	38	18.5(15-20)	
No result*****	117	46	13	9	

Table 3.2: Clinical features of of paediatric cases and controls*RTHC: Road to Health Card

** Pearson chi square

***** Either failed to return for Mantoux reading or not documented in patient's clinical notes

3.2.2 Paediatric Controls

A cohort of healthy, age matched children from the same neighbourhood, living within three streets of, but not in the same household as a TB case, was recruited as a control group. All control children were Mantoux skin tested and results documented. Any child with an induration diameter of 15mm or greater was referred to the local TB clinic for a chest X ray and assessment to determine whether or not active TB was present, in accordance with national guidelines and excluded from the study if diagnosed with TB.

The cohort of healthy children was recruited to the study for two main purposes:

- To enable comparison of the functional immunological phenotype in healthy children with that measured in past history TB cases
- To provide a cohort of healthy children for future use in case-control studies testing newly identified polymorphisms in candidate genes for their role in TB susceptibility

All controls were seen and bled twice at an interval of 2 to 4 weeks to check for stability of the functional immunological phenotype.

3.2.3 Adult Controls

A further cohort of 756 healthy adult controls with no history of TB disease, from the Xhosa and Coloured communities were recruited from the same environment as the paediatric controls and cases. Local church communities and factory workers were invited to donate a 10ml sample of blood after screening to exclude prior TB disease or treatment for latent TB infection. Because no functional phenotype was measured and a prior history of TB was excluded, no screening for

HIV infection was considered necessary. It was assumed that all adult controls living in a TB endemic environment had been exposed to and infected with *M. tb*.

The healthy adult controls consisted of 82.4% Xhosa, 17.6% Coloured subjects.

3.3 Screening the promoter region of the IL12p40 in cases and controls

Sequence variations in genes of the IFN γ pathway were identified using two different techniques. The Imperial College London group used the technique of Mutation Detection Gel Analysis (MDGA) from Laboratoires Ellios Biotec for screening the IFN γ R1, IL12p40, IL12p35, IL12R β 2 genes. For the work in this thesis I used the WAVE technique for screening of the IL12p40 promoter, as apparatus for MDGA was not available in Cape Town. These two methods enabled large scale, mutation screening of candidate genes to be completed.

To ensure that the two methods were comparable, a validation of the WAVE technique was done (see section 3.4.1 below).

3.3.1 Methodology

3.3.1.1 MDGA

MDGA is a combination of two electrophoretic DNA separation techniques: single stranded conformation polymorphism (SSCP) and heteroduplex mobility analysis (HMA), using PCR amplicons (Crepin *et al.* 2003). During electrophoresis, double stranded DNA moves through a gel at a rate that is determined by its nature and length. Single strands in contrast are affected by any base change, as without a complementary strand complementary nucleotides will pair with each

other, forming a unique structure of loops and folds dependent on the sequence. The presence of SNPs results in different secondary structures, which on electrophoresis show different migration patterns. SSCP utilizes this latter property to identify mutations and polymorphisms in PCR amplified DNA. In this technique, double stranded DNA is denatured and immediately cooled on ice, preventing reannealing of the double strands and allowing folding of the single strands into their secondary structures.

HMA is similar to SSCP but the test sample and wild type are mixed, denatured and allowed to reanneal to form a heteroduplex. A mutation in one of the strands will cause a mismatch in the heteroduplex, with an alteration in conformation and electrophoretic migration pattern.

MDGA has the advantage of combining electrophoretic discrimination of both single stranded DNA secondary structure and double stranded homoduplex/heteroduplex. Thus it has the potential to improve on resolution of SSCP and HMA used individually. This is shown in figure 3.1. Additionally, MDGA has an advantage of operating optimally at one temperature.

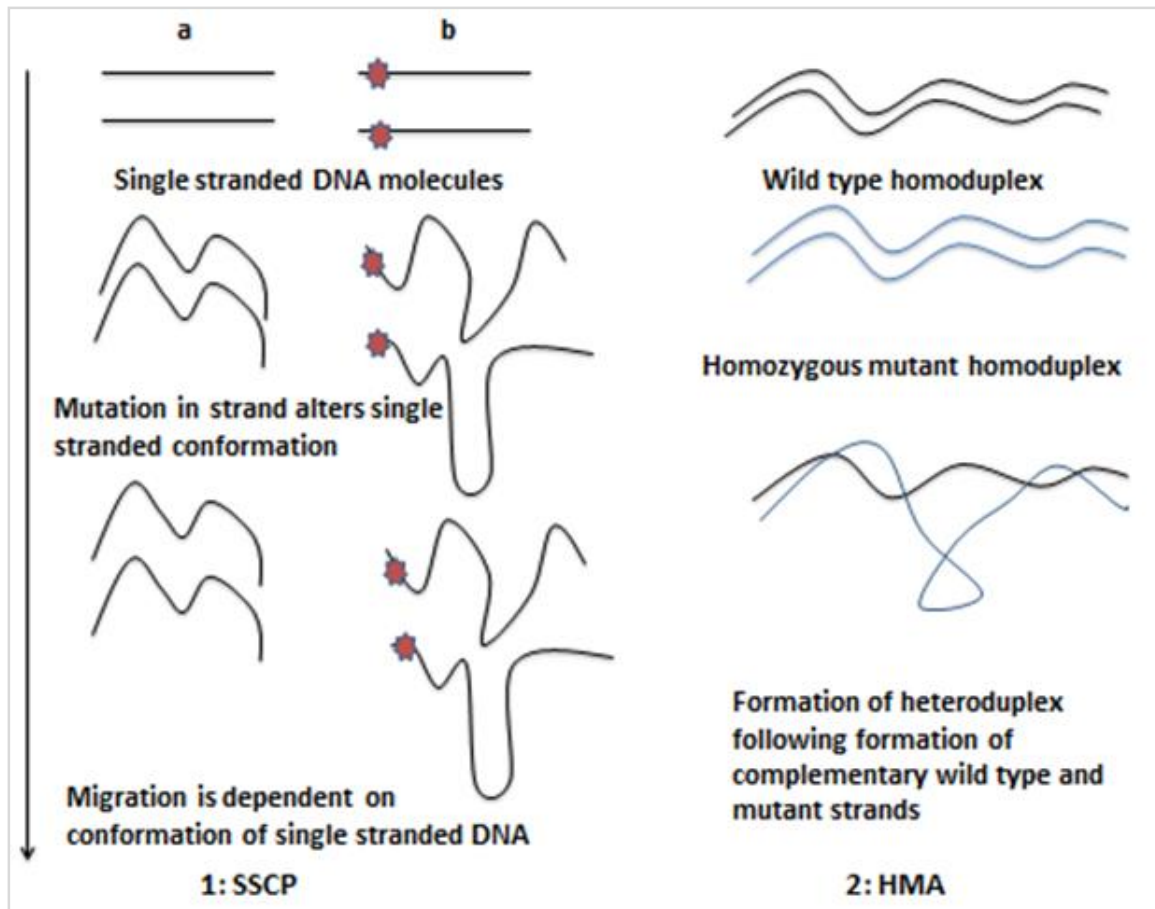


Figure 3.1: Principles of SSCP and HMA techniques that are combined in the MDGA technique.

In example 1 (SSCP) a mutation in strand b is shown to result in different secondary structure and migration pattern.

In example 2 (HMA) a homozygous mutant results in the formation of a bubble in the heteroduplex when the strands are allowed to re-anneal, retarding migration on the gel.

3.3.1.2 WAVE

WAVE utilizes the properties of MDGA with denaturing high performance liquid chromatography (DHPLC) and is able to distinguish between two or more DNA sequences.

Heteroduplexes are prepared by mixing the PCR amplified “normal” DNA from a healthy control and “test” DNA samples for each test mutation. If the normal and patient PCR product sequences are identical, a homoduplex forms. If the patient DNA sequence is different to the normal, a heteroduplex forms. These heteroduplexes are run on the WAVE instrument, allowing for the detection of mutations by the differential retention of homoduplex and heteroduplex amplicons on reversed-phase chromatography supports. These supports are housed in heated columns where the operational temperatures are derived by computational analysis of the DNA sequence (Oefner PJ *et al.* 2000, Xiao *et al.* 2001).

When running the samples on the WAVE instrument, a normal control homoduplex sample is always included with test samples, for comparison of elution patterns. Any migration pattern different from the normal sample is considered as a possible sequence change and the genomic DNA of that region of the gene is sequenced. This is depicted in figure 3.2 below.

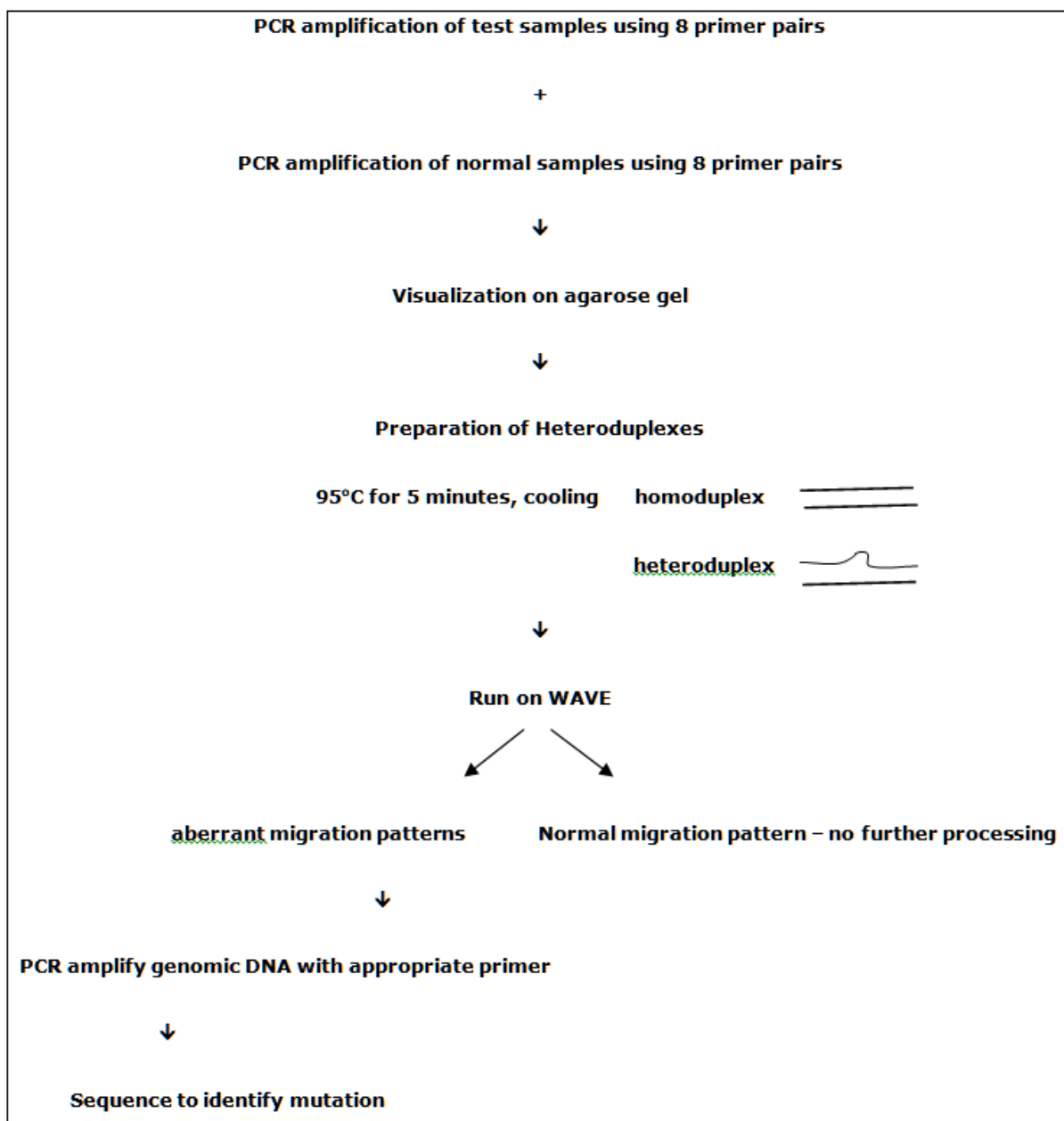


Figure 3.2: Flow diagram of procedure for WAVE screening for DNA changes.

The WAVE and MDGA techniques are very similar as regards their underlying DNA chemistries, cost requirements, mutation detection sensitivities and the use of 96 well plates. However, the main technical difference is that while MDGA uses a single electrophoretic

temperature for one sample, WAVE may use more than one temperature for each sample, depending of the nature of the DNA sequence. Different sequences of DNA fragments result in individual conformations which shape uniquely at different temperatures. Thus, for all amplicons run on the WAVE instrument, the melting domains of the PCR product fragments are calculated by sequence computation prior to running the samples.

A comparison of the MDGE and WAVE mutation detection methods is summarized in table 3.3.

MDGA technique	WAVE technique
PCR product run on ABI 377 sequencer using Elliomut gel™	PCR products run on WAVE Nucleic Acid Fragment Analysis System
DNA amplicons run on gel, with molecular weight markers, detected by fluorescence	DNA amplicons bound to column, eluted and detected by UV after Denaturing High Performance Liquid Chromatography
Fluorescent dCTPS used (yellow, green, blue)	Standard dNTPS used
Fluorescent PCR products fade with time	PCR products stable
Single injection of sample, single electrophoretic temperature	Melting profiles of amplicons calculated before running, sometimes more than one injection required if more than one DNA domain
Amplicons with mutations migrate at different rates to those of normal sequences	Amplicons with mutations forming heteroduplexes elute at different times to homoduplexes of normal sequences.

Table 3.3: Comparison of the WAVE and MDGA techniques

3.4 Methods and Results

3.4.1 WAVE validation

To ensure that the two methods were comparable, a validation of the WAVE technique was completed using three genomic DNA samples with known mutations previously identified by the MDGA technique in London.

Genomic DNA from three samples with known mutations in the IFN γ R1 promoter (TT deletion), IL12R β 2 (C \rightarrow T SNP) and TNFR1 (C \rightarrow T SNP) genes, previously identified using MDGA, were screened by WAVE. In addition DNA from a healthy donor was PCR amplified with appropriate primers. Heteroduplexes were prepared by mixing the normal and mutant DNA samples for each test mutation and run on the WAVE instrument.

All three test mutations were successfully detected by WAVE. Results are shown in figures 3.3, 3.4 and 3.5.

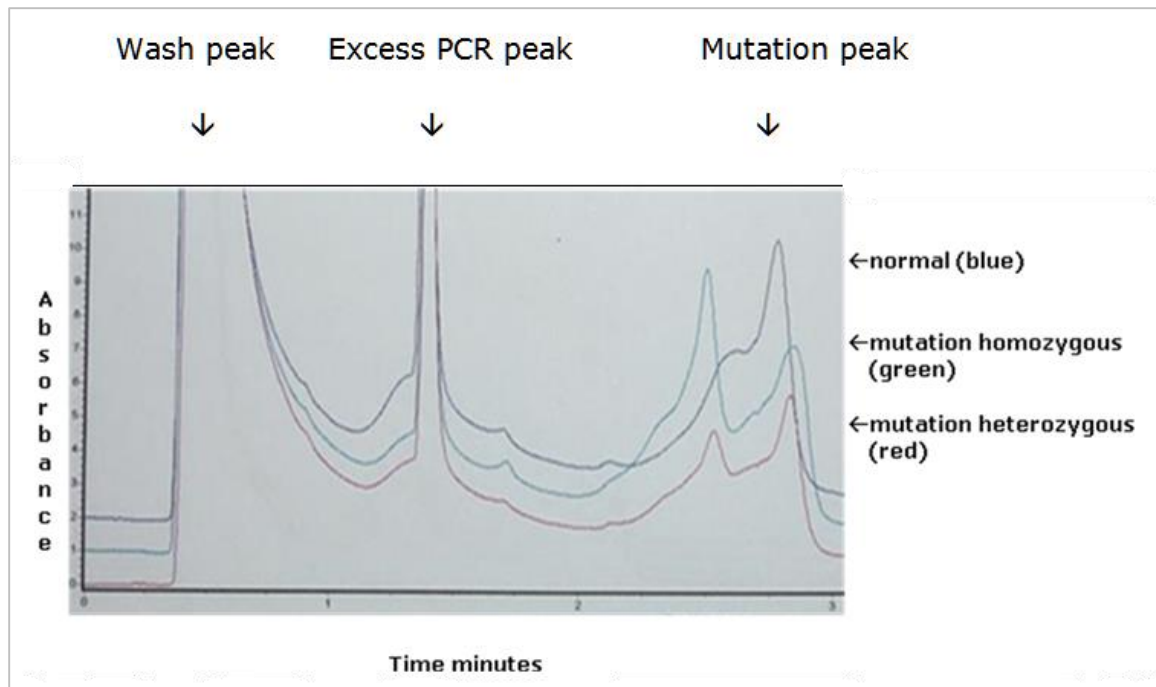


Figure 3.3: Heteroduplex WAVE trace of test mutation 1, TT deletion in the IF γ R1 promoter and normal sequence, showing the different conformation determined elution times: homozygous TT deletion (green), heterozygote TT deletion (red), normal sequence (blue).

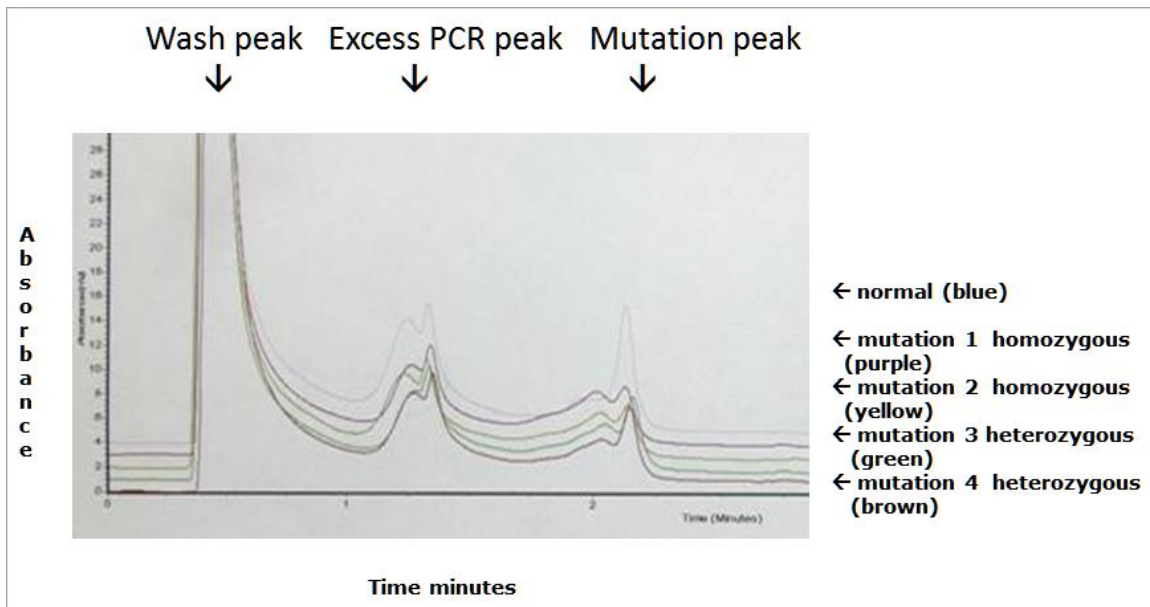


Figure 3.4: Heteroduplex WAVE traces of test mutation 2, C→T SNP in the IL12R β 2 gene in two patients and normal sequence, showing the different conformation determined elution times: Patient 1 homozygous T/T (purple), patient 2 homozygous T/T (yellow), patient 3 heterozygote C/T (green), patient 4 heterozygote C/T (brown), normal sequence C/C (blue).

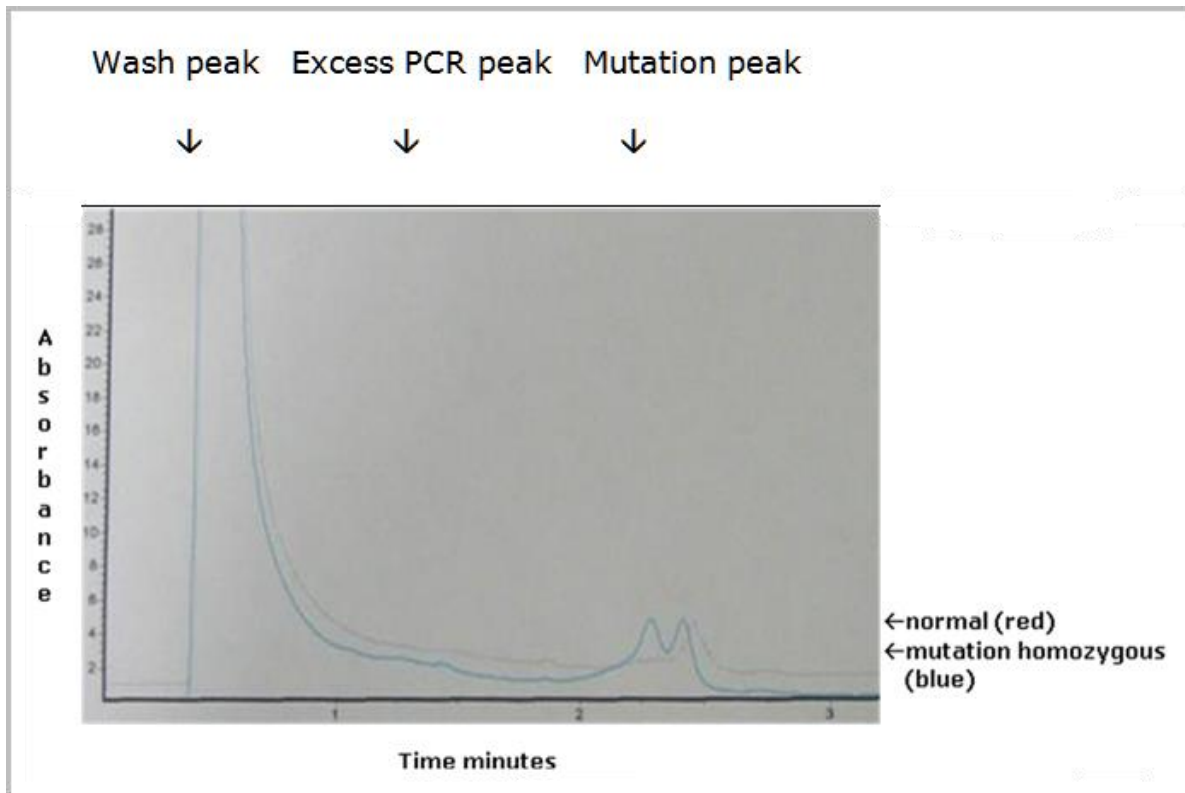


Figure 3.5: Heteroduplex WAVE traces of test mutation 3, C→T SNP in the TNFR1 gene and normal sequence, showing the different conformation determined elution times: homozygous T/T (blue), normal sequence C/C (red).

3.4.2 Screening of study samples

After successful validation of the method, screening for DNA changes in the IL12p40 promoter was carried out by the WAVE method. Because of limited resources and the longer processing time of WAVE compared to MDGA, screening of a limited number of randomly selected paediatric samples was completed first, followed by ARMS-PCR to screen for any SNPS identified in the remaining paediatric cohort and healthy adult control samples.

The screening process is shown in figure 3.6.

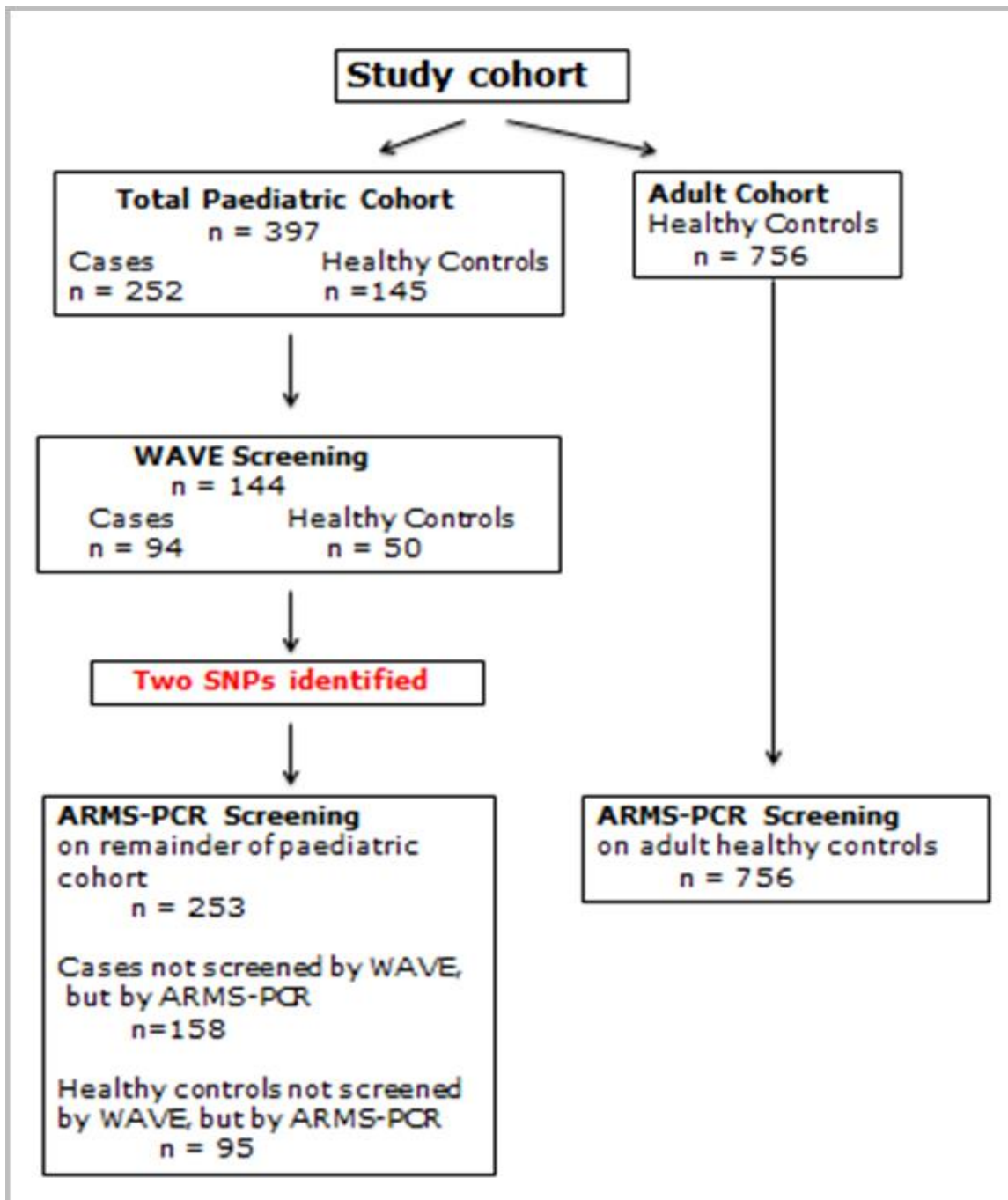


Figure 3.6: Algorithm showing screening of IL12p40 promoter by WAVE and ARMS-PCR.

The categories of the paediatric TB cases screened by WAVE are shown in table 3.4.

	Active TB	Past History TB	Total
Definite	12	13	25
Highly Probable	6	0	6
Probable	29	5	34
Possible	18	11	29
Total	65	29	94

Table 3.4: Categories of paediatric TB cases screened by WAVE

The categories of all paediatric TB cases screened by ARMS-PCR are shown in table 3.5.

	Active TB	Past History TB	Total
Definite	27	23	49
Highly Probable	0	3	3
Probable	61	13	74
Possible	15	16	31
Total	103	55	158

Table 3.5: Categories of all paediatric TB cases screened by ARMS-PCR

3.4.2.1 WAVE screening of study samples

3.4.2.2 Amplification of IL12p40 promoter for WAVE screening

The section of the IL12p40 promoter chosen for study extended from +73 to -1949 (2022bp). This section of the promoter sequence was used as it encompassed the following transcription factor binding sites: TATAbox at -28, C/EBP at -80, NFkB at -115, PU.1 at -126, GA-12 at -162, ets at -211, AP-1 at -232, SP-1 at -350, NF-IL6 at -510, IRF-1 at -728, as shown in figure 2.1 and described in chapter 2.

The fragment of the IL12p40 promoter was amplified using eight overlapping pairs of primers. The eight primer pairs were designed by Dr Suzanne Anderson to generate amplicons of 300-400bp, the optimum size for WAVE screening. These are listed with the respective melting points in table 3.6.

Primer	Sequence 5' to 3'	Amplicon size bp	Melting temp °C
IL12BP1F	AAGCTTCTTTTGCATAACTGGC	300	55,57,60.5
IL12BP1R	CAACTGGGGGAGGGGCTTT		
IL12BP2F	AACTACTTCACGTCAATTTCTG	339	56,58.5,61.5
IL12BP2R	CTGGCCGTGGGTGGAGAC		
IL12BP3F	AGGCCTAGAGGACACAGGG	344	65.1
IL12BP3R	CGCAGGTCCACTTCTCCATC		
IL12BP4F	TAAAACCCGGCCTGGTCCC	337	65.5
IL12BP4R	GGAATTTCAAGTTGCTAAGAGG		
IL12BP5F	ACATGTTCTGTTCACGTGCA	291	60.8
IL12BP5R	GTGCTCATAGGTACTCGCTC		
IL12BP6F	CTGTATGCCTCCCTGAGGG	332	58.1, 59.5
IL12BP6R	CCTGGTTCTTCCCAAGTCAG		
IL12BP7F	CCTTTGCCCTTCATCTCATCCT	389	57,61
IL12BP7R	TTGCAACATTGAAAACAACTCTC		
IL12BP8F	TTTTGATGTCTATGTTCCCTCC	259	57.3,59.1,60.8
IL12BP8R	TTGGGAAGTGCTTACCTTGCT		

Table 3.6: Amplicon melting temperatures and primers for amplification of the IL12p40 promoter for WAVE

For WAVE screening, eight PCR products of each test DNA were prepared. At the same time, eight PCR products of a normal sample from a healthy control were prepared for generation of heteroduplexes. PCR products were visualized on a 1% agarose gel to check for clarity and intensity prior to WAVE analysis.

Heteroduplexes were prepared by mixing equal volumes of “normal” PCR product with “test” PCR product (for example 12 μ l+12 μ l of equivalent gel intensity PCR product), incubating at 95°C for 5 minutes in a thermal cycler and then switching the cycler off, allowing gradual cooling for 45 minutes.

After running on the WAVE instrument, migration patterns were compared and all samples with aberrant patterns were sequenced for DNA changes.

3.4.2.3 WAVE screening results

Potential sequence changes were identified in several of the PCR products amplified using the second of the overlapping primer pairs BP2F and BP2R shown in table 3.6.

This region of genomic DNA of all these samples was PCR amplified and sequenced for identification of the mutations.

3.4.2.4 Sequencing for identification of mutations detected by WAVE

Sequencing reaction was done using the Big Dye® Terminator Cycle Sequencing kit by myself and electrophoresis of sequenced samples carried out at the Department of Human Genetics, UCT.

In total, only two heterozygous SNPs, 42 bp apart, were identified by the WAVE screening, 6 samples had an adenine for guanine

substitution at position -1523 (-1523 A→G) and 8 samples had a cytosine for thymine substitution at position -1564 (-1564 C→T).

Wave migration patterns of the PCR heteroduplex samples are shown in figures 3.7 and 3.8.

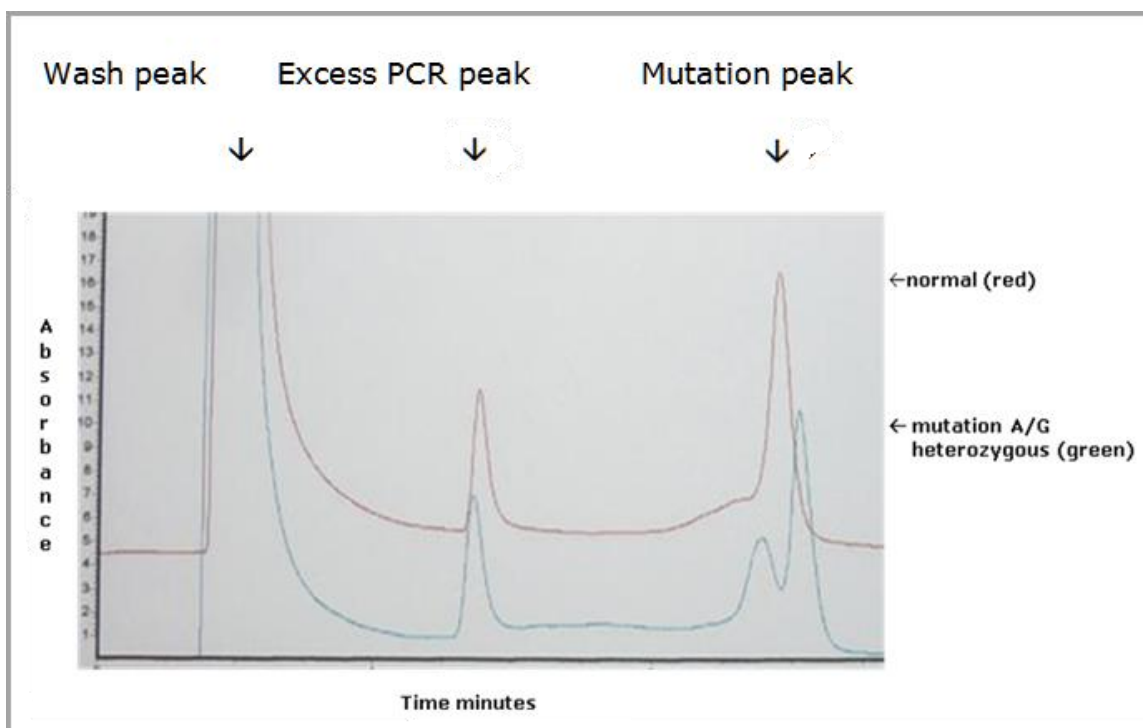


Figure 3.7: Heteroduplex WAVE trace of normal DNA and SNP - 1523 A/G showing the differing conformation determined elution times: heterozygote A/G (green), normal sequence A/A (red).

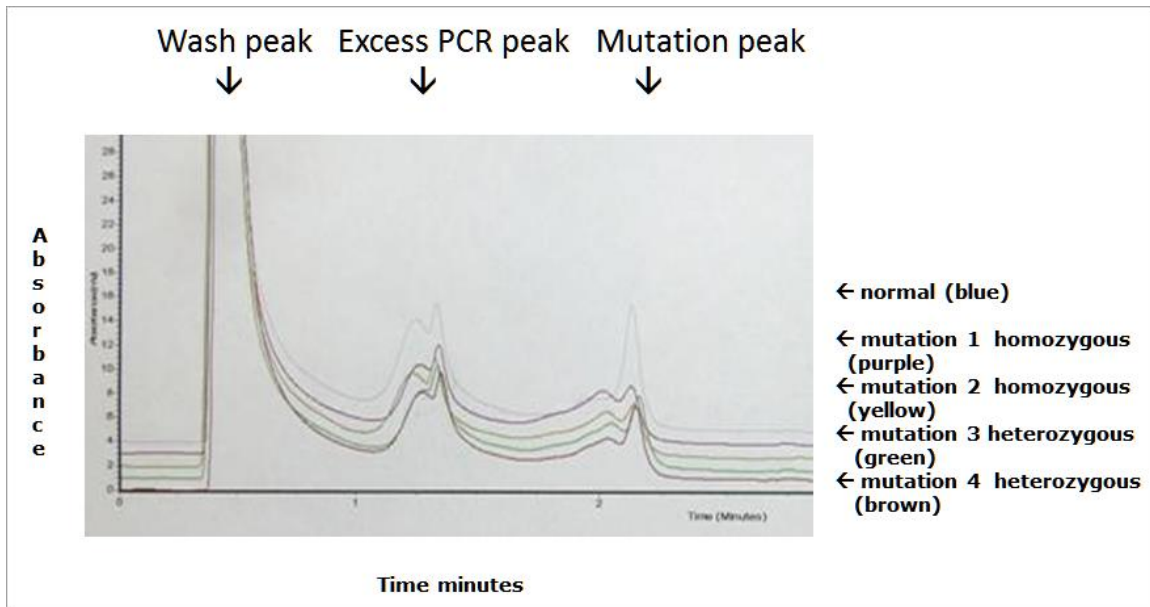


Figure 3.8: Heteroduplex WAVE trace of normal DNA and SNP -1564 C/T showing the differing conformation determined elution times: heterozygote C/T (yellow), normal sequence C/C (red).

Sequence traces of the genomic DNA SNP -1523 A/G and SNP -1564 C/T heterozygotes are shown in figures 3.9 and 3.10.

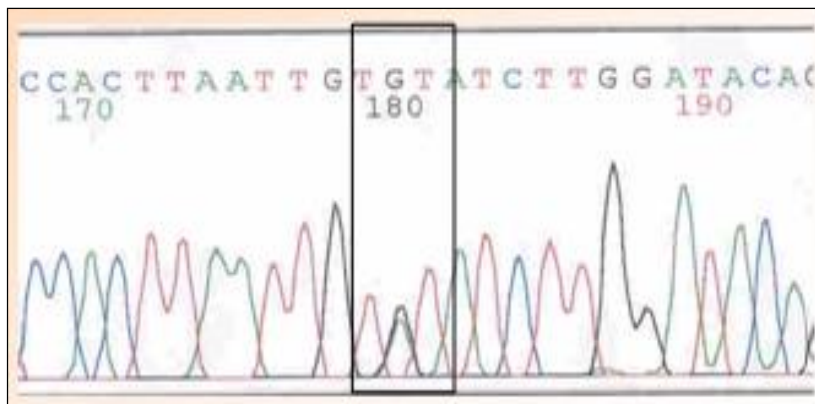


Figure 3.9: Sequence trace of genomic DNA, showing the SNP -1523 A/G in the heterozygous state.

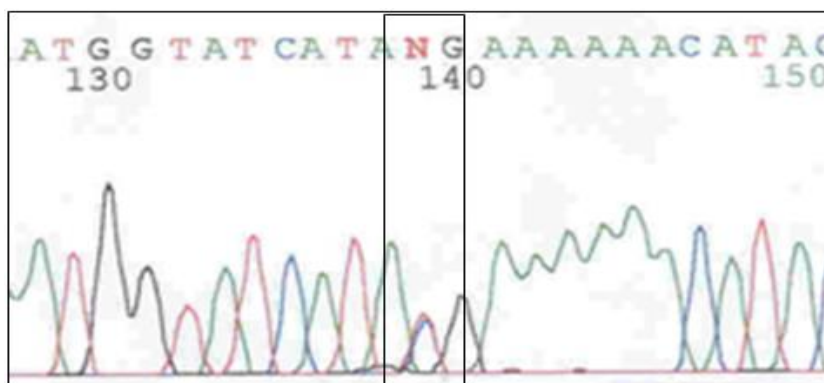


Figure 3.10: Sequence trace of genomic DNA, showing the SNP -1564 C/T in the heterozygous state.

3.5 ARMS-PCR screening of the larger cohort for the -1523 A/G and -1564 C/T SNPs

As these two SNPs were initially identified in a subset of the cohort, the remainder of the samples therefore required screening.

Screening for the presence of the two SNPs in the remaining samples was completed using Amplification Refractory Mutation System PCR (ARMS-PCR). This methodology is used for detection of heterozygotes by amplifying both alleles in one PCR tube. These unique ARMS-PCR assays were designed and optimized by myself for this wider screening.

3.5.1 Methods and results

Two allele specific, mismatched forward primers and one reverse primer were used for each of the ARMS-PCR assays. For the SNP -1523 A/G and SNP -1564 C/T ARMS-PCR reactions, the WAVE primer pair IL12BP2F, IL12BP2R was used with additional allele specific, forward mismatched primers, ARMS-Fg and ARMS-Ft that would amplify only the G and T alleles respectively. Primer positions and amplicon sizes for the ARMS-PCR assays are depicted in figure 3.11.

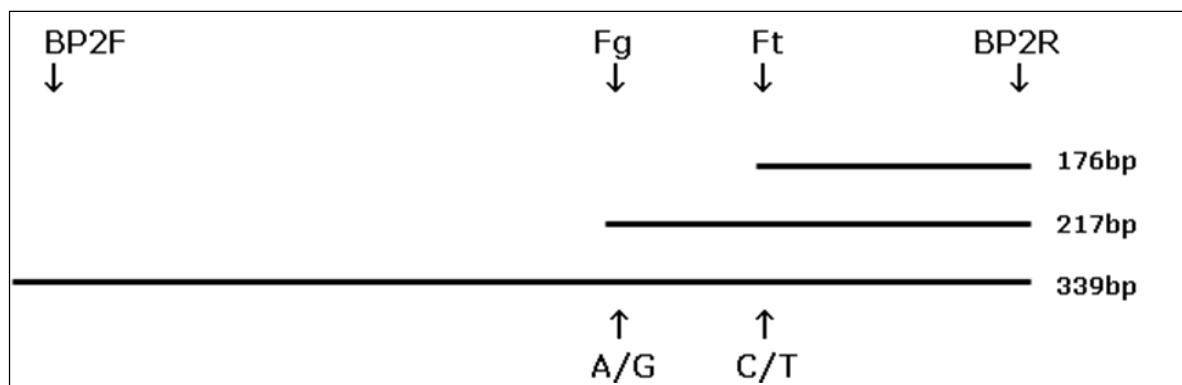


Figure 3.11: Schematic representation of primer annealing areas and amplicons generated by the ARMS-PCR assay, showing allele specific mismatched primers Fg and Ft for detection of G and T alleles.

Amplification of the A/A genotype resulted in two identical PCR fragments 339bp long, while amplification of the A/G genotype resulted in two PCR products of 339 and 217bp respectively.

Amplification of the C/C genotype resulted in two identical PCR fragments 339bp long while amplification of the C/T genotype resulted in two PCR products of 339 and 176bp respectively.

Unique additional forward primers used for the novel ARMS-PCR assays were designed. The ARMS-Fg primer ends in G for detection of G allele and ARMS-Ft primer ends in T for detection of T allele. These primers have single base mismatches near the 3' end, highlighted in Table 3.7. As three primers are used in one tube in the ARMS-PCRs, the primer sequences were designed to have similar thermodynamic temperatures (Tms). PCR conditions were optimized and the concentrations of the two forward primers and common reverse primer adjusted for the single tube assay. The concentrations of the two forward primers were optimized to yield bands of similar

intensities. The common reverse primer was used at a higher concentration than that of the forward primers.

Primer	Sequence 5' to 3' – mismatched bases in bold
ARMS-Fg	AATCTGCTTTGTCCACTTAATT CTG (-1523 A/G)
ARMS-Ft	CACCCCACAAATGGTATCA GAT (-1564C/T)
IL12BP2F	AACTACTTCACGTCAATTTCTG
IL12BP2R	CTGGCCGTGGGTGGAGAC

Table 3.7: Allele specific mismatched primers for ARMS-PCR for detection of -1523 A/G and -1564 C/T SNPs

3.5.1.1 ARMS-PCR optimization

The ARMS-PCR was first optimized using different concentrations of primers, until intensities of all amplified alleles were similar. Ethidium bromide stained agarose gels (3%) of PCR products of the two assays are shown in figure 3.12.

If no amplicons were seen on the agarose gel, the ARMS-PCR was repeated. All positive SNPs detected were confirmed by repeat ARMS-PCR.

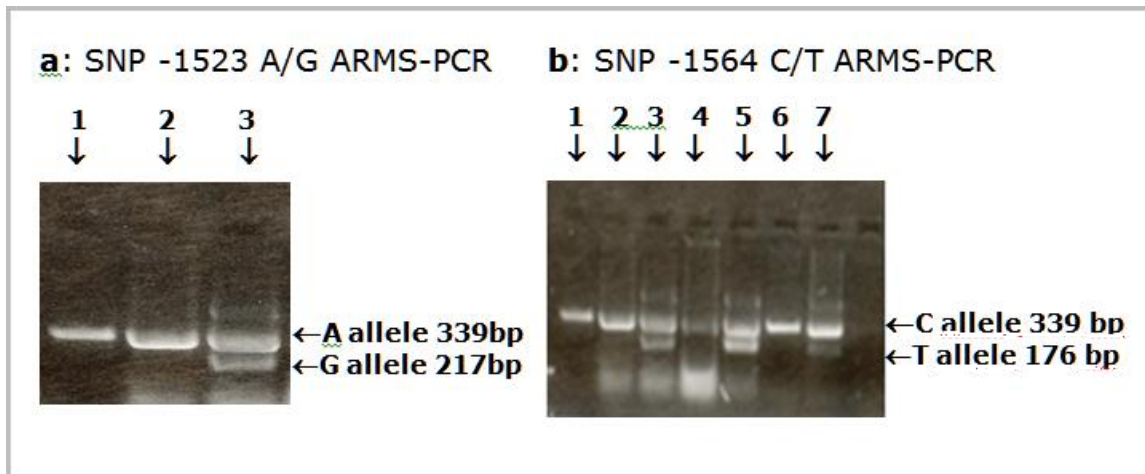


Figure 3.12: Agarose gel (3%) electrophoresis of ARMS-PCR products.

a: Lane 1, Marker 339bp; lane 2, homozygous A/A genotype 339bp; lane 3, heterozygous A/G genotype 339bp and 217bp

b: Lane 1, Marker 339bp; lanes 2,4, 6 homozygous C/C genotype 339bp; lanes 3, 5, 7 heterozygous C/T genotype 339bp and 176bp

Further screening was then done for the two SNPs in the remaining paediatric sample (cases $n = 158$ and controls $n = 95$) (total $n = 426$) and adult control samples ($n = 756$).

Large scale screening for the SNPs -1523 A/G and -1564 C/T was done using 96 well plates. PCR products were electrophoresed on large 3% agarose gels, ethidium bromide stained and visualized under UV light for detection of the G and T alleles, shown in figures 3.13 and 3.14.

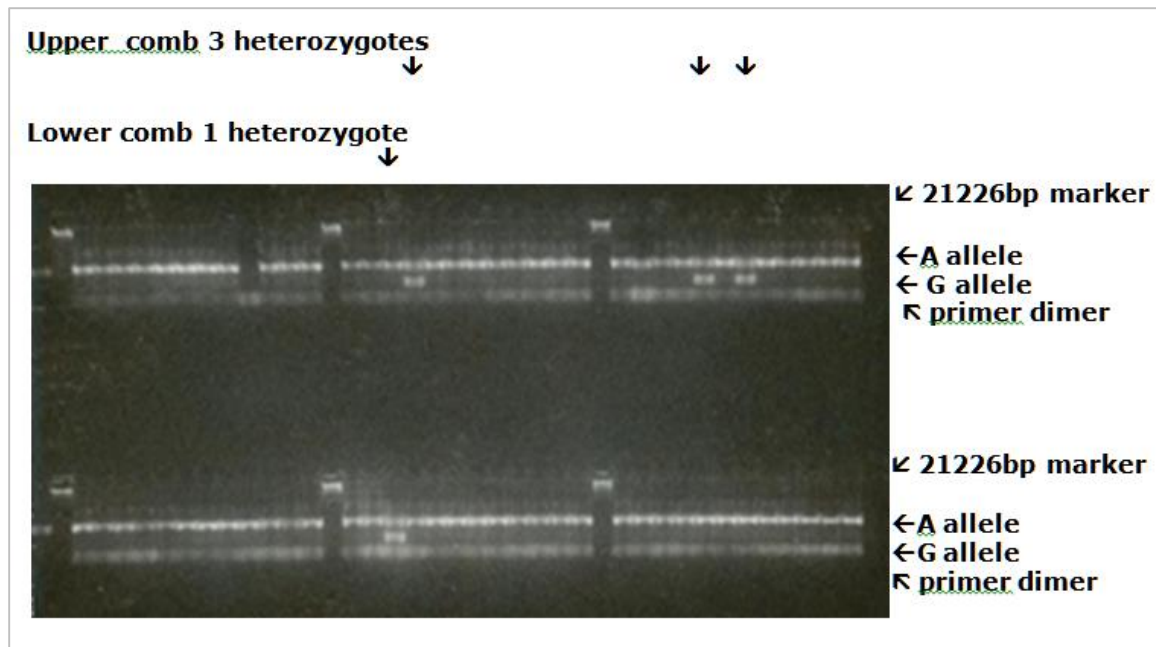


Figure 3.13: Agarose gel (3%) electrophoresis of ARMS-PCR products for detection of -1523 A/G SNP. Upper comb shows 21226bp marker and three DNA samples heterozygous for both A and G alleles. Lower comb shows 21226bp marker and one DNA sample heterozygous for both A and G alleles.

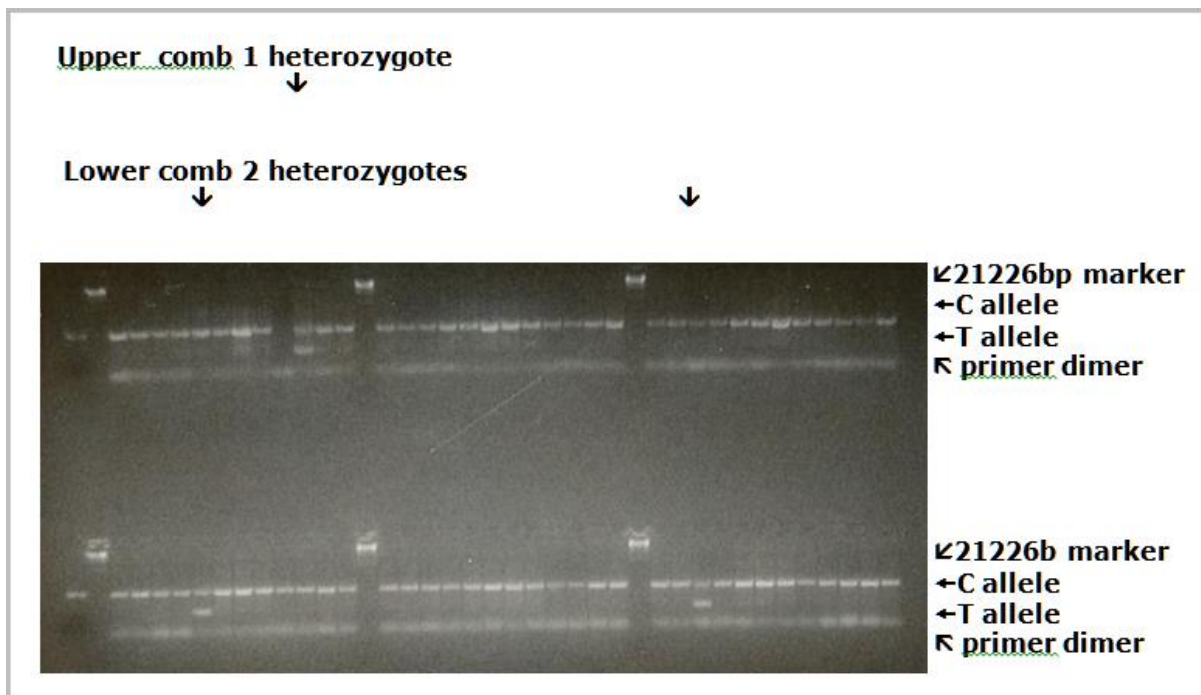


Figure 3.14: Agarose gel (3%) electrophoresis of ARMS-PCR products for detection of -1564 C/T SNP. Upper comb shows 21226bp marker and one DNA sample heterozygous for both C and T alleles. Lower comb shows 21226bp marker and two DNA samples heterozygous for both C and T alleles.

After the extended screening, in total, 61 samples (cases n=12, controls n=49) were found to have the adenine for guanine substitution at position -1523 (-1523 A→G) and 60 samples (cases n=20, controls n=40) were found to have the cytosine for thymine substitution at position -1564 (-1564 C→T).

3.6 Submission to SNP database

The two SNPs were submitted to the SNP database and given the following "submitter SNP" numbers:

AGHETEROZYGOTE 107795940

CTHETEROZYGOTE 107795941

3.7 Statistical Analysis

Statistical analysis was done using STATA 9

Initially, comparisons of frequencies of the two SNPs between cases and controls was carried out on the subcohort of children screened by WAVE, results shown in tables 3.8 and 3.9. Thereafter, the expanded paediatric group screened by WAVE and ARMS-PCR was analysed.

To increase the power of the study, ARMS-PCR was done on an expanded group, using adult control samples.

Results were not stratified using ethnicity since the majority of paediatric subjects screened were 90% Xhosa and 10% Coloured (paediatric subjects) and 80% Xhosa and 20% Coloured (adult subjects).

Differences between cases and controls combining Xhosa and Coloured groups into one group were investigated for each SNP by Fischer's exact and Pearson chi-square tests.

3.7.1 Statistical analysis of the small paediatric cohort screened by WAVE

Genotype	TB Cases (%)	Controls (%)	Total (%)
A/G	1 (1.1%)	5 (10.0%)	6 (4.2%)
A/A	93 (98.9%)	45 (90.0%)	138 (95.8%)
Total	94 (100.0%)	50 (100.0%)	144 (100.0%)

Table 3.8: Frequencies of the SNP -1523 A/G and SNP -1523 A/A genotypes in the paediatric subcohort screened by WAVE

Notes:

Fischer's exact $p = 0.02$

-1523 A/G Odds Ratio (OR) 0.1 (0.002 – 0.92)

Genotype	TB Cases (%)	Controls (%)	Total (%)
C/T	5 (5.3%)	3 (6.0%)	8 (5.6%)
C/C	89 (94.7%)	47 (94.0%)	136 (94.4%)
Total	94 (100.0%)	50 (100.0%)	144 (100.0%)

Table 3.9: Frequencies of the SNP -1564 C/T and SNP -1564 C/C genotypes in the paediatric subcohort screened by WAVE.

Notes:

Fischer's exact $p = 1.0$

-1564 C/T Odds Ratio 0.9 (0.16 – 5.91)

The frequencies of the A/G and A/A genotypes recorded in table 3.8 showed the A/G SNP to be significantly less prevalent in TB cases compared to controls [1.1% vs 10%]; (Fischer's exact $p=0.02$; $n=94$ and 50 respectively).

The statistically significant p value of 0.02 for the A/G and A/A genotypes in this small paediatric group, suggested that the risk of TB

disease is significantly reduced in those subjects with the -1523 A/G genotype compared to those with the -1523 A/A genotype.

3.7.2 Statistical analysis of larger paediatric cohort of children screened by WAVE and ARMS-PCR

The number of children screened for these promoter SNP frequencies was increased to test the resilience of the earlier findings. The results are shown in tables 3.10 and 3.11.

Genotype	TB Cases (%)	Controls (%)	Total (%)
A/G	12 (4.8%)	10 (6.9%)	22 (5.5%)
A/A	240 (95.2%)	135 (93.1%)	375 (94.5%)
Total	252 (100%)	145 (100%)	397 (100%)

Table 3.10: Frequencies of the SNP -1523 A/G and SNP -1523 A/A genotypes in the larger group of paediatric subjects screened by WAVE and ARMS-PCR

Notes:

Pearson chi-square = 0.801 p = 0.4

-1523 A/G Odds Ratio 0.7 (0.26 -1.80)

Genotype	TB Cases (%)	Controls (%)	Total (%)
C/T	20 (7.9%)	9 (6.2%)	29 (7.3%)
C/C	232 (92.1%)	136 (93.8%)	368 (92.7%)
Total	252 (100%)	145 (100%)	397 (100%)

Table 3.11: Frequencies of the SNP -1564 C/T and SNP -1564 C/C genotypes in the larger group of paediatric subjects screened by WAVE and ARMS-PCR

Note:

Pearson chi-square = 0.407 p = 0.5

-1564 C/T Odds Ratio 1.3 (0.55 - 3.34)

In this expanded cohort, neither the frequencies of A/G and A/A genotypes nor those of the C/T and C/C genotypes in paediatric TB cases and controls, recorded in tables 3.10 and 3.11 respectively, were significantly different.

3.7.3 Statistical analysis of all paediatric cases and controls, including adult controls

Because of the small number of paediatric controls, healthy adult controls were added to the control group. Results are shown in tables 3.12 and 3.13.

Genotype	TB Cases (%)	Controls (%)	Total (%)
A/G	12 (4.8%)	49 (5.4%)	61 (5.3%)
A/A	240 (95.2%)	852 (94.6%)	1092 (94.7%)
Total	252 (100%)	901 (100%)	1153 (100%)

Table 3.12: Frequencies of the SNP -1523 A/G and SNP -1523 A/A genotypes in the expanded group of paediatric subjects and healthy controls (paediatric and adult)

Notes:

Pearson chi-square = 0.180 p = 0.7

-1523 A/G Odds Ratio 0.9 (0.41 - 1.69)

Genotype	TB Cases (%)	Controls (%)	Total (%)
C/T	20 (7.9%)	40 (4.4%)	60 (5.2%)
C/C	232 (92.1%)	861 (95.6%)	1093 (94.8%)
Total	252 (100%)	901 (100%)	1182 (100%)

Table 3.13: Frequencies of the SNP -1564 C/T and SNP -1564 C/C genotypes in the expanded group of paediatric cases and healthy controls (paediatric and adult)

Notes:

Pearson chi-square = 4.882 p = 0.03

-1564 C/T Odds Ratio (OR) 1.9 (1.0 – 3.3)

In this expanded cohort, the frequencies of A/G and A/A genotypes in paediatric TB cases and controls recorded in table 3.12 were not significantly different.

However, the frequencies of C/T and C/C genotypes recorded in table 3.13, showed that the -1564 C/T SNP was significantly more prevalent in TB cases compared to controls [7.9% vs 4.4%; (Fischer's exact p = 0.03); n = 252 and 901 respectively].

The p value of 0.03 in the expanded group suggested that the C/T genotype may be associated with susceptibility to TB, as it was observed at a significantly higher frequency in TB cases.

Analysis was repeated on SNP frequencies, excluding the category of "Possible TB" cases, but this did not show any significance differences between cases and controls.

3.7.4 Concluding statement

Screening the IL12p40 promoter for sequence variants among a cohort of paediatric TB cases and healthy controls identified two SNPs. This

finding suggests that the IL12p40 promoter is highly conserved. However, a number of methodological reasons may have accounted for this apparent lack of genetic variation. The initial WAVE screening was restricted to 144 DNA samples, a subset of the whole cohort that was chosen randomly. The rationale for this approach was logistical since the method is very time consuming and does not lend itself to the high throughput screening of a large number of samples. If the entire cohort had been screened by this method more SNPs may have been identified. However, given the time and financial constraint this was not feasible.

An alternative approach that could have been used would have been to complete the initial screening on an enriched cohort of cases with severe disease, including TBM, miliary TB or those with low IL12/IFN γ responses, on the basis that these cases were more likely to harbour functional mutations.

Another significant limitation was related to the small size of the paediatric cohort and especially the number of culture confirmed, 'definite' TB cases. One of the challenges of completing work on paediatric TB is that the gold-standard of culture confirmed disease is rarely achieved, adding a level of uncertainty to the categorisation of cases. This is probably the main reasons why so few genetic studies of paediatric TB are reported in the literature. It is therefore possible that some of the cases categorised as probable and possible TB, and included in this analysis, had another disease.

The paediatric control group was also small and for this reason a healthy adult control cohort, with no history of TB disease, was recruited and included in this study. Controls were recruited from the same communities as cases and, because all were living in a TB

endemic environment, it was assumed that all were infected with *M. tb*. When the adult control group was included, analysis of the -1523 A/G and -1564 C/T SNPs showed that there was a significant difference between cases and controls for the -1564 C/T SNP, which was more prevalent in the cases.

Increasing the control group with adult samples has the advantage of detecting the effect of a weak SNP. However, detrimental mutations may also have been lost in the adult population, due to death earlier in life, resulting in a falsely significant difference between paediatric cases and adult controls.

The -1523 A/G SNP occurred within a pentanucleotide sequence (GT**A**TATA), reported to bind nuclear factors. A previous report of the GTATA sequence in the HLA-DR α gene, showed that by gel retardation assays, a synthetic double stranded oligonucleotide containing both GTATA and IFN γ consensus sequences was able to form specific complexes with nuclear factors from cell lines of different histotypes (Barbieri *et al.* in 1990). Amaya *et al.* 2001 studied promoter recognition in vegetative bacteria and reported the GTATA consensus sequences were recognized by sporulation associated σ factors. These observations are unrelated to the IL12p40 promoter, but there was nevertheless the possibility of this rare pentanucleotide being important in the expression of the IL12p40 gene.

This was the rationale for testing for a functional role in determining susceptibility to disease. This is detailed in chapter 4.

CHAPTER 4: Functional assessment of IL12p40 promoter SNPs

4.1 Introduction

The functional consequences of promoter sequence variations, on gene expression, are commonly evaluated by reporter assay systems.

The reporter system of choice for this thesis was the Dual-Luciferase® Reporter assay, where the activities of firefly (*Photinus pyralis*) and sea pansy (*Renilla reniformis*) luciferases are measured sequentially in a single test sample. It is possible to selectively discriminate between their respective bioluminescent reactions as the enzymes have differing substrate requirements.

Reporter assays are carried out on cell homogenates that have been transfected with DNA constructs. The DNA is ligated into the cloning site of an expression vector that carries the gene for firefly luciferase, with the cloned DNA insert acting as the promoter for the luciferase gene. Cells are co-transfected with an expression vector that carries the promoter and coding sequence of the sea pansy luciferase. The latter high copy number construct, used as an internal control, serves as a baseline response.

The mouse macrophage cell line, RAW 264.7, was chosen for this study as good results have been obtained by other groups using the Dual-Luciferase® Reporter Assay System. For limited comparative purposes, the human monocyte THP-1 and human fibroblast HT1080 cell lines, were also used. The expression vectors used for the reporter assay were the pGL3-Basic vector (firefly luciferase) and the pRL-TK vector with the thymidine kinase promoter (sea pansy

luciferase), gifts from Dr. Sharon Prince, Department of Human Biology, Medical School, University of Cape Town.

This chapter describes the cloning of the PCR amplified promoter inserts into the pGEM®-T vector and sub-cloning into the pGL3-Basic expression vector. The following strategy was used for the preparation and cloning of the normal, SNP -1523 G, SNP -1564 T promoter fragments and two truncation/deletion promoter control fragments.

4.2 Methods and Results

4.2.1 The IL12p40 promoter constructs

The section of the IL12p40 promoter chosen for investigation extended from +73 to -1949 (2022bp). This section was selected as it encompassed the following transcription factor binding sites: TATAbox at -28, c/EBP at -80, NFkB at -115, Pu.1 at -126, GA-12 at -162, ets at -211, AP-1 at -232, SP-1 at -350, NF-IL6 at -510, IRF-1 at -728. Several early publications defined the existence and importance of these transcription factor binding sequences, shown in figure 4.1 as discussed in chapter 2 (Murphy *et al.* 1995, Ma *et al.* 1996, Ma *et al.* 1997, Gri *et al.* 1998 and Becker *et al.* 2001).

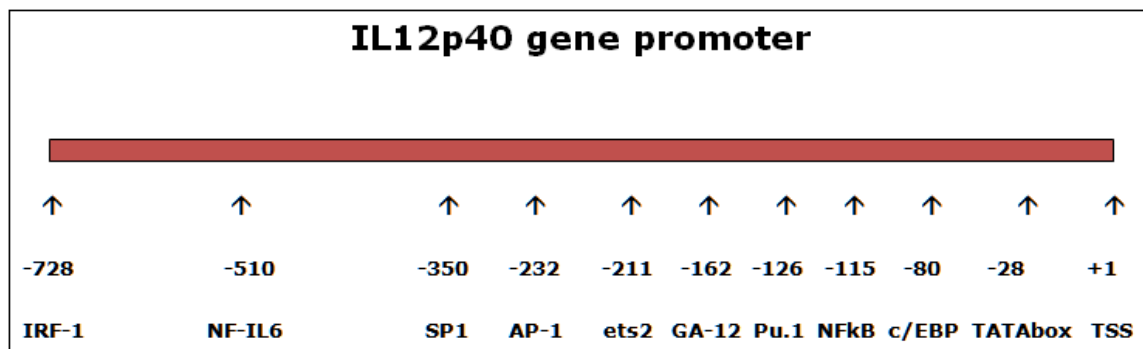


Figure 4.1: Transcription factor binding sites of the human IL12p40 gene promoter.

As detailed in chapter 3, two heterozygous promoter SNPs were detected in the patient cohort studied, these being an A→G substitution at nucleotide position -1523 (-1523 A/G) and a C→T substitution at -1564 (-1564 C/T). Three 2022bp long, full sequence promoter constructs were therefore required for the reporter assay. 1) A promoter section carrying the normal sequence at positions -1523 (A) and -1564 (C), 2) A section carrying the -1523 (G) SNP with the normal -1564 (C) sequence and 3) a section carrying the normal -1523 (A) sequence and the -1564 (T) SNP sequence. Two negative or null controls were also needed for the reporter assay. It was thus decided to prepare two promoter segments missing critical regulatory sequences. This was achieved through truncation and deletion of sequence.

4.2.2 Preparation of the Normal, SNP -1523 G and SNP -1564 T promoter constructs

The promoter variants were prepared by PCR using uniquely adapted primers. In order to clone PCR products it is necessary to include vector compatible restriction enzyme cutting sites into the forward and reverse primers used to generate the PCR fragments. The 5' *SacI* and 3' *XhoI* cutting sites of the pGL3-Basic expression vector were chosen for sub-cloning in this study.

The adapted PCR primers used for RE cutting site inclusion were the forward primer IL12BP1F (*SacI*) and reverse primer IL12BP8R (*XhoI*). These primers were adapted from those previously used for the WAVE screening described in chapter 3.

To facilitate digestion of the 5' ends of the PCR products, the tagged on 6bp cutting sequences were extended by 3 random bases to allow

for RE binding. The respective 9bp 5' sequences incorporated into the IL12BP1F and IL12BP8R primers, are shown in table 4.1.

Primer	Sequences with 9bp 5' extensions	Incorporated restriction site
Original P1F	AAGCTTCTTTTGCATAACTGGC	
Adapted P1F	ACA <u>GAGCTC</u> CTTTTGCATAACTGGC	<i>GAGCT'C</i> <i>SacI</i>
Original P8R	TTGGGAAGTGCTTACCTTGCT	
Adapted P8R	ATA <u>CTCGAG</u> GGAAGTGCTTACCTTGCT	<i>C'TCGAG</i> <i>XhoI</i>

Table 4.1: Adapted primers used for PCR of normal, SNP -1523 G and SNP -1564 T promoter sequences

4.2.3 Cloning of promoter variants into T vector system

The normal and SNP -1523 G and SNP -1564 T promoter sequences were first cloned into a T vector system. This is necessary as *Taq* polymerase adds an A base onto the 3' end of the amplicons. The 5' T vector system thus facilitates ligation and cloning through A=T complementary binding.

Several tubes of PCR product of each of the three promoter fragments to be cloned (normal, SNP -1523 G and SNP -1564 T), were pooled respectively and cleaned on a spin column.

These three promoter fragments were ligated into the pGEM®-T Easy Vector (figure 4.2), (supplied linearized) and used to transform competent cells. The transformation cultures were grown on LB/amp/IPTG/Xgal agar plates and screened for inserts through blue/white colony selection.

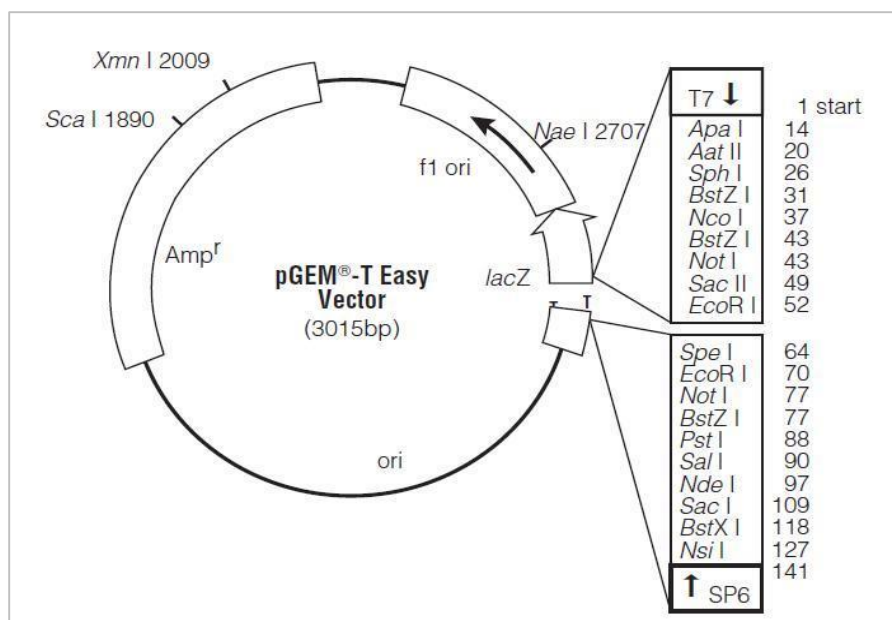


Figure 4.2: pGEM®-T Easy Vector circle map and multiple cloning site.

The pGEM®-T vector used in blue/white colony screening, contains the β lactamase gene, which confers ampicillin resistance on the plasmid, thereby permitting only the growth of competent cells transformed with plasmids on medium supplemented with the antibiotic. The F1 origin of replication and the *lacZ* gene in the pGEM®-T vector allow for plasmid replication and *lacZ* gene expression. The *lacZ* gene specifies synthesis of part of the β -galactosidase enzyme and this gene is very near the multiple cloning site of the plasmid. Cells harbouring normal plasmids would be ampicillin resistant and able to synthesize β -galactosidase. Cells harbouring recombinant plasmids, with DNA cloned into them, would be ampicillin resistant, but unable to synthesize β -galactosidase, due to the disruption of the *lacZ* gene sequence by the inserted DNA fragment. IPTG (isopropyl-thiogalactoside) is an inducer of the *lac* genes and ensures that the *lacZ* expression is not repressed. X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) is a substrate for β -galactosidase and is broken

down by the enzyme into a blue coloured product. By plating bacterial cells transformed with plasmids onto agar containing ampicillin, IPTG and X-gal, the recombinant colonies containing the inserted DNA are distinguishable by their white colour.

White colonies were picked and screened for the presence of inserts by PCR, using any pair of the original WAVE oligonucleotides, generating amplicons of approximately 300bp in size. Master plates of colonies with inserts were prepared. Overnight cultures were grown and plasmid DNA extracted from colonies with inserts of the correct size.

Colonies with inserts were checked for any PCR errors by sequencing of plasmid DNA minipreps. This was done to ensure that any results observed from the functional assays would be solely as a result of the variant DNA changes and not PCR errors. DNA changes were interpreted as true mutations when identified in more than one sequenced clone.

An ethidium bromide agarose gel of PCR screening of white colonies is shown in figure 4.3.

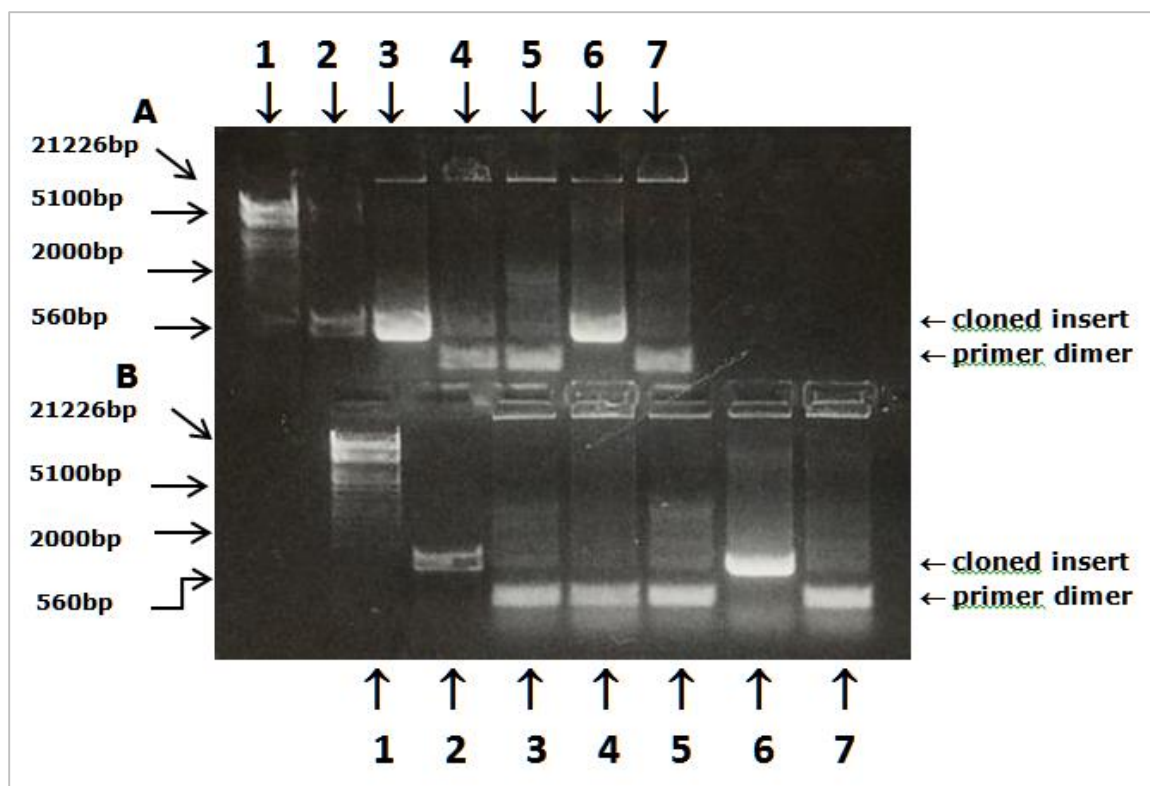


Figure 4.3: Agarose gel (1%) gel electrophoresis of PCR screening of colonies for cloned inserts.

Notes:

Comb A: Lane 1 λ H/E markers (21226bp-560bp); lane 2, marker (339bp)(slightly degraded); lanes 3 and 6, inserts; lanes 4, 5, 7, no inserts.

Comb B: Lane 1 λ H/E markers (21226bp-300bp); lane 2, marker (339bp)(slightly degraded); lane 6, insert; lanes 4, 5, 7, no inserts.

An amplicon of 339bp generated from the WAVE screening was used as a low molecular weight marker. Due to extensive use, it became slightly degraded over time.

In spite of using Hi Fidelity *Taq* polymerase, PCR errors were observed in many of the cloned inserts. Sequencing showed that about 90% of clones had 1 or 2 PCR errors, which occurred randomly. PCR errors are a well-recognized occurrence. Reports on the fidelity of several thermostable DNA polymerases has shown that mononucleotide and

dinucleotide repeats are not faithfully reproduced by PCR and that most commercially available *Taq* polymerases introduce errors at a rate of approximately 10^{-5} to 10^{-7} point mutations/bp/duplication. High fidelity polymerases generate up to eight times fewer errors (Cline *et al.* 1996, Clarke *et al.* 2001).

Eventually normal and SNP -1564 T clones, free of PCR errors, were confirmed. Overnight cultures were grown for storage at -80°C and plasmid DNA minipreps prepared.

All sequence traces were compared to normal genomic sequences lodged in the genome database. Comparisons were generated by the National Centre for Biotechnology Information (NCBI) blast alignment search tool.

A sequence trace and NCBI blast alignment of the cloned normal promoter are shown in figures 4.4 and 4.5.

Query	96	TGTCTTATTTTATGCATGTATTTATTTATgggggggCACAAGGACTCATCTGTGGTGGTG	155
Sbjct	3162	TGTCTTATTTTATGCATGTATTTATTTATGGGGGGGCACAAGGACTCATCTGTGGTGGTG	3221
Query	156	CAGCCACTGTAAATAAATTAGTGAAACTACTTCACGTCAATTTCTGTTCACTACACTTTA	215
Sbjct	3222	CAGCCACTGTAAATAAATTAGTGAAACTACTTCACGTCAATTTCTGTTCACTACACTTTA	3281
Query	216	GTGATGGATCGGAGGAAATTAATACATGTTTACAAAAGCCCCTCCCCAGTTGTTACAT	275
Sbjct	3282	GTGATGGATCGGAGGAAATTAATACATGTTTACAAAAGCCCCTCCCCAGTTGTTACAT	3341
Query	276	ATGCCTCAGAGATACCAGTTGTGAAAAGTGCAGGTGCACTTACACACATACGCACACACA	335
Sbjct	3342	ATGCCTCAGAGATACCAGTTGTGAAAAGTGCAGGTGCACTTACACACATACGCACACACA	3401
Query	336	CCCCACAAATGGTATCATACGAAAAAACATACCTGCAATCTGCTTTGTCCACTTAATTGT	395
Sbjct	3402	CCCCACAAATGGTATCATACGAAAAAACATACCTGCAATCTGCTTTGTCCACTTAATTGT	3461
Query	396	ATATCTTGATACAGAACTTGTTTCACTGGAAGGCTAAAAGGCAAAGTCTGGGGAGGCCT	455
Sbjct	3462	ATATCTTGATACAGAACTTGTTTCACTGGAAGGCTAAAAGGCAAAGTCTGGGGAGGCCT	3521

Figure 4.5: Comparison of normal cloned fragment and normal genomic sequence on NCBI blast alignment search tool. Boxes show C and A bases of normal sequence. Boxes in this figure correspond to arrows in figure 4.4 above.

A sequence trace and NCBI blast alignment of the cloned SNP -1564 T fragment are shown in figures 4.6 and 4.7.

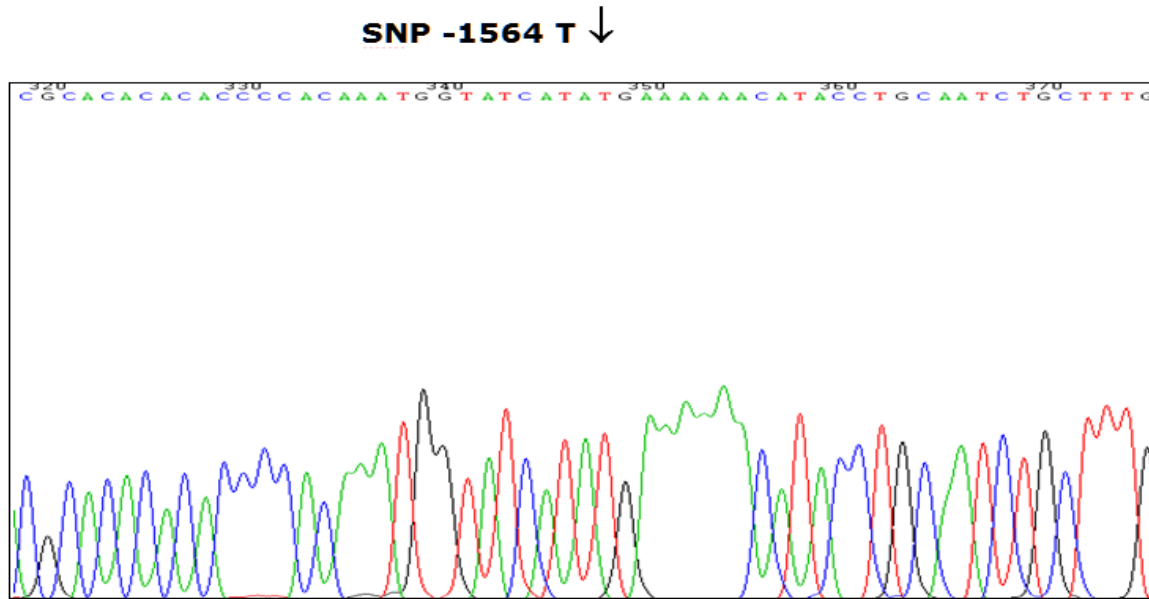


Figure 4.6: A section of the sequence trace of cloned SNP -1564 T fragment.

Query	97	TGTCCTATTTTATGCAATGATTTATTTATgggggggCACAAGGACTCATCTGTGGTGGTG	15
Sbjct	3162	TGTCCTATTTTATGCAATGATTTATTTATGGGGGGGCACAAGGACTCATCTGTGGTGGTG	32
Query	157	CAGCCACTGTAAATAAATTAGTGAAACTACTTCACGTCAATTTCTGTTTCAGTACACTTTA	21
Sbjct	3222	CAGCCACTGTAAATAAATTAGTGAAACTACTTCACGTCAATTTCTGTTTCAGTACACTTTA	32
Query	217	GTGATGGATCGGAGGAAATTAATACATGTTTACAAAAAGCCCCTCCCCCAGTTGTTACAT	27
Sbjct	3282	GTGATGGATCGGAGGAAATTAATACATGTTTACAAAAAGCCCCTCCCCCAGTTGTTACAT	33
Query	277	ATGCCTCAGAGATACCAGTTGTGAAAAGTGCAGGTGCACTTACACACATACGCACACACA	33
Sbjct	3342	ATGCCTCAGAGATACCAGTTGTGAAAAGTGCAGGTGCACTTACACACATACGCACACACA	34
Query	337	CCCCACAAATGGTATCATATGAAAAACATACCTGCAATCTGCTTTGTCCACTTAATTGT	35
Sbjct	3402	CCCCACAAATGGTATCATACGAAAAACATACCTGCAATCTGCTTTGTCCACTTAATTGT	34
Query	397	ATATCTTGGATACAGAACTTGTTCCTGGAAGGCTAAAAGGCAAAGTCTGGGGAGGCCT	45
Sbjct	3462	ATATCTTGGATACAGAACTTGTTCCTGGAAGGCTAAAAGGCAAAGTCTGGGGAGGCCT	35

Figure 4.7: Comparison of cloned SNP -1564 T and normal sequences on NCBI blast alignment search tool. Box shows SNP -1564 T. Box in this figure corresponds with arrow in figure 4.6 above.

4.2.4 Digestion of normal and SNP -1564 T fragments out of T vector plasmid

The normal and SNP -1564 T fragments were digested out of the pGEM®T vector plasmid using *Sac*I and *Xho*I restriction enzymes. After double digestion, the fragments were electrophoresed on an agarose gel, viewed under long wave UV, excised and extracted. An ethidium bromide stained agarose gel of a cloned fragment released from the T vector is shown in figure 4.8.

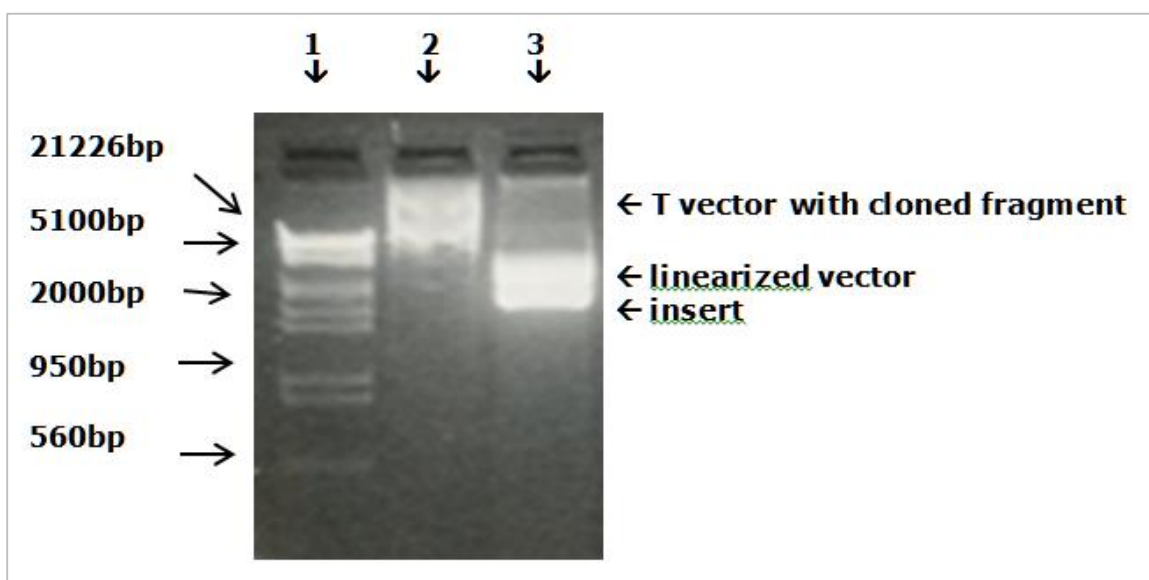


Figure 4.8: Agarose gel (1%) electrophoresis showing T vector with cloned fragment and released fragment. Lane 1 λ H/E markers (21226bp-560bp); lane 2, vector with cloned fragment; lane 3, fragment released by digestion.

Unfortunately, after extensive screening, no PCR error free SNP -1523 G clones were found, necessitating a different approach to its preparation.

4.2.5 New approach to SNP -1523 G fragment generation.

The problem of PCR errors in the SNP -1523 G clones was circumvented by ligating the PCR error free fragment from a normal clone (AG4) with that from the flawed SNP -1523 G clone (AG44) which carried an error T-511C. These individual PCR error free sections were generated by digesting both purified inserts with *Sma*I, which cut the promoter at one restriction site only, at nt -1287. This gave selective fragments of 663bp and 1359bp free of PCR error. These fragments were purified by electrophoresis and gel extraction. The 1359bp fragment from the PCR error free normal clone (AG4) was ligated to the 663bp fragment from the SNP -1523 G clone (AG44) creating a SNP -1523 G fragment free of any PCR errors. This strategy is depicted in figure 4.9.

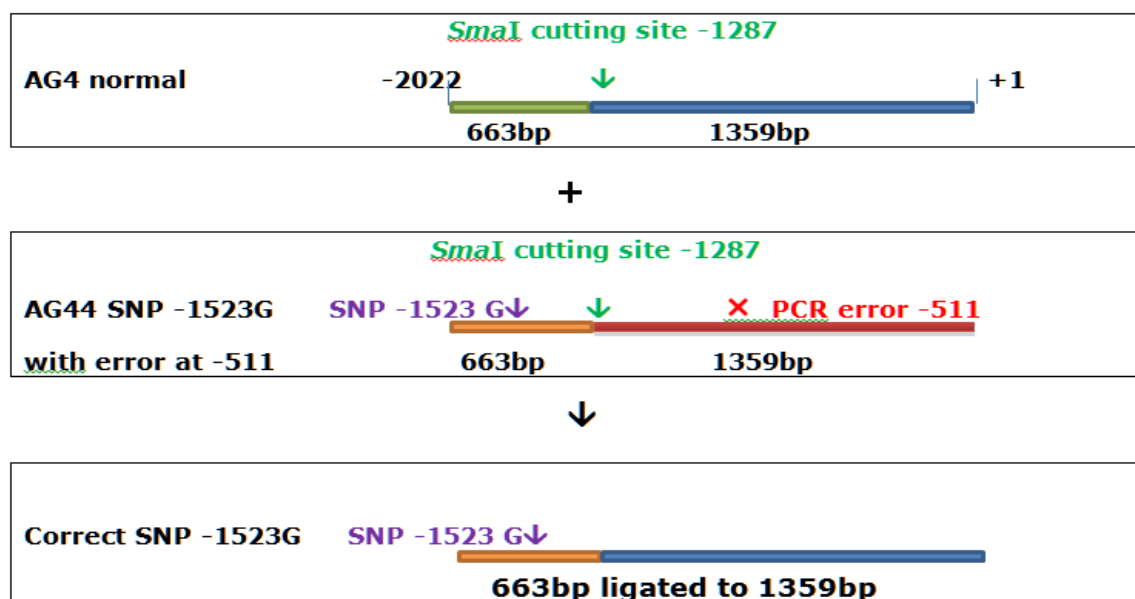


Figure 4.9: Schematic representation of the generation of the SNP -1523 G fragment through ligation of PCR error free sequences from AG44 (663bp) and AG4 (1359bp) after digestion with SmaI CCC▼GGG at nt -1287.

An ethidium bromide stained agarose gel of the *Sma*I digests of the AG44 and AG4 fragments is shown in figure 4.10.

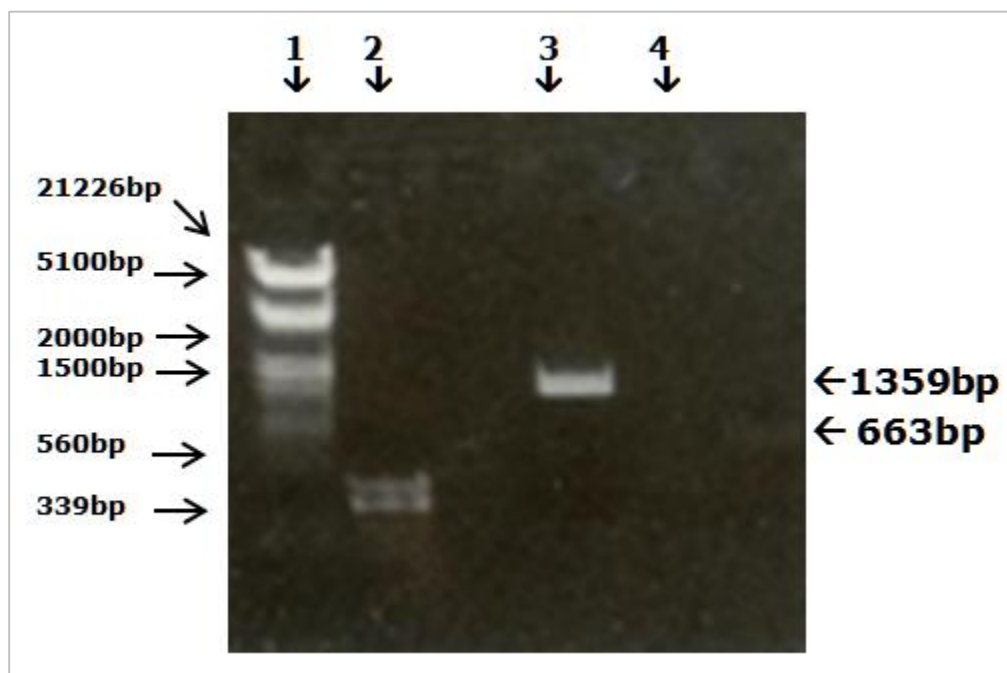


Figure 4.10: Agarose gel (1%) electrophoresis of *Sma*I digests of AG4 (normal) and AG44 (SNP -1523 G clone with PCR error at nt -511). Lane 1 λ H/E markers (21226bp-560bp); lane 2, marker (339bp marker, slightly degraded); lane 3, digested AG4 (1359bp fragment); lane 4, digested AG44 (663bp fragment).

The 663bp fragment in lane 4 is faint, as only a small amount was available for electrophoresis. Most was used for ligation and cloning.

These two fragments were ligated to create a PCR error free SNP - 1523 G promoter fragment and sub-cloned directly into the pGL3-Basic expression vector as described later.

4.2.6 Creation of the truncated and deletion IL12p40 promoter constructs.

Two additional constructs were created as negative controls for the reporter assay. One was a truncated promoter with the TATAbox, c/EBP, NFkB, Pu.1 and GA-12 response elements missing and the other was a promoter with essential elements TATAbox, c/EBP, NFkB, Pu.1, GA-12 and ets deleted.

4.2.6.1 Creation of the truncated construct

The truncated promoter was created by amplifying 1744bp of the plasmid DNA miniprep of the normal promoter from -1949 (1952 with adapted primer) to -208. In order to enable this construct to be cloned into the pGL3-Basic expression vector, the initial adapted forward primer with *SacI* restriction site and overhang was used and a new reverse primer with the *XhoI* restriction site and overhang designed for the amplification by PCR. These primers are listed in table 4.2.

Primer	Sequence with 9bp 5' extension	Incorporated restriction site
Adapted P1F	ACAGAGCTCCTTTTGCATAACTGGC	GAGCT'C <i>SacI</i>
Adapted R truncated	ATACTCGAGGGTTTACATCATGGCCA	C'TCGAG <i>XhoI</i>

Table 4.2: Primers used for creation of truncated promoter

The truncated 1744bp construct was missing the TATAbox, c/EBP, NFkB, Pu.1 and GA-12 response elements as shown in figure 4.11.

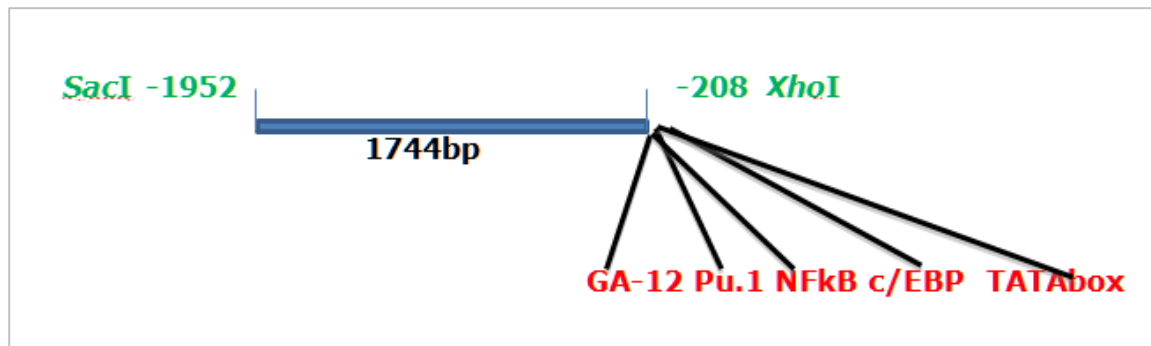


Figure 4.11: Truncated promoter construct 1744bp in length, showing the absence of TATAbox, c/EBP, NFkB, Pu.1 and GA-12 transcription factor binding sequences.

Several tubes of PCR product of the amplified truncated promoter were pooled and cleaned on a spin column.

An ethidium bromide stained agarose gel of the PCR product of the truncated promoter is shown in figure 4.12.

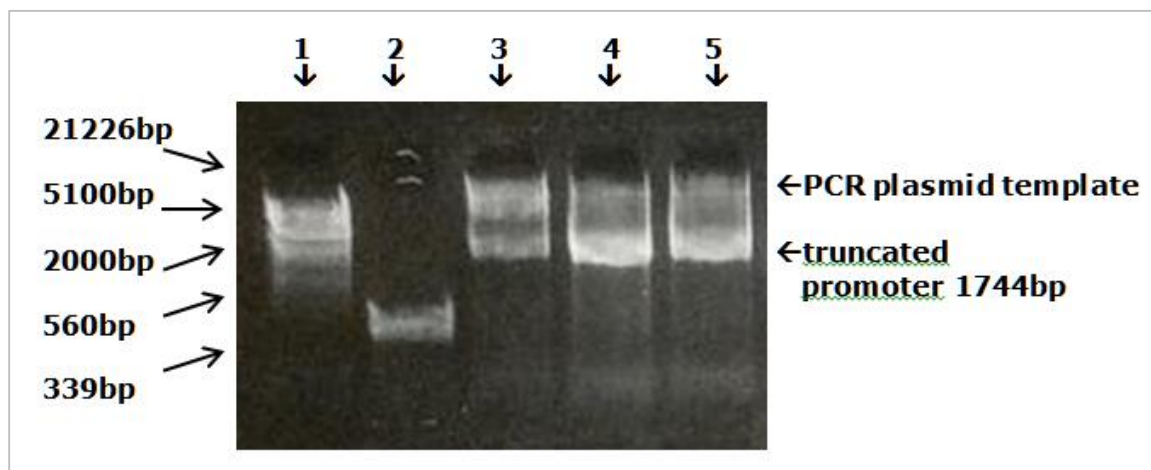


Figure 4.12: Agarose (1%) gel electrophoresis of PCR product of truncated promoter. Lane 1 λ H/E markers (21226bp-560bp); lane 2, marker (339bp marker, slightly degraded); lanes 3-5, truncated promoter (1744bp) from 3 PCR reactions.

4.2.6.2 Creation of the deletion construct

The full length 2031bp normal promoter from +73 (+79 with adapted primer) to -1949 (1952 with adapted primer) was amplified and digested with *Ba*I, at a single site, nt -275. This generated two fragments of 1673bp and 358bp in length. This strategy was used as *Ba*I was a blunt cutter which allows for ligation of PCR fragments. The 1673bp fragment was then ligated to the 3' end of the 173bp fragment, which had been created by amplification of the normal promoter from -94 to +73 (+79 with adapted primer). This resulted in a deletion construct of 1848bp in length with transcription factors TATAbox, c/EBP, NFkB, Pu.1, GA-12 and ets deleted. The strategy for creation of the deletion construct is shown in figure 4.13

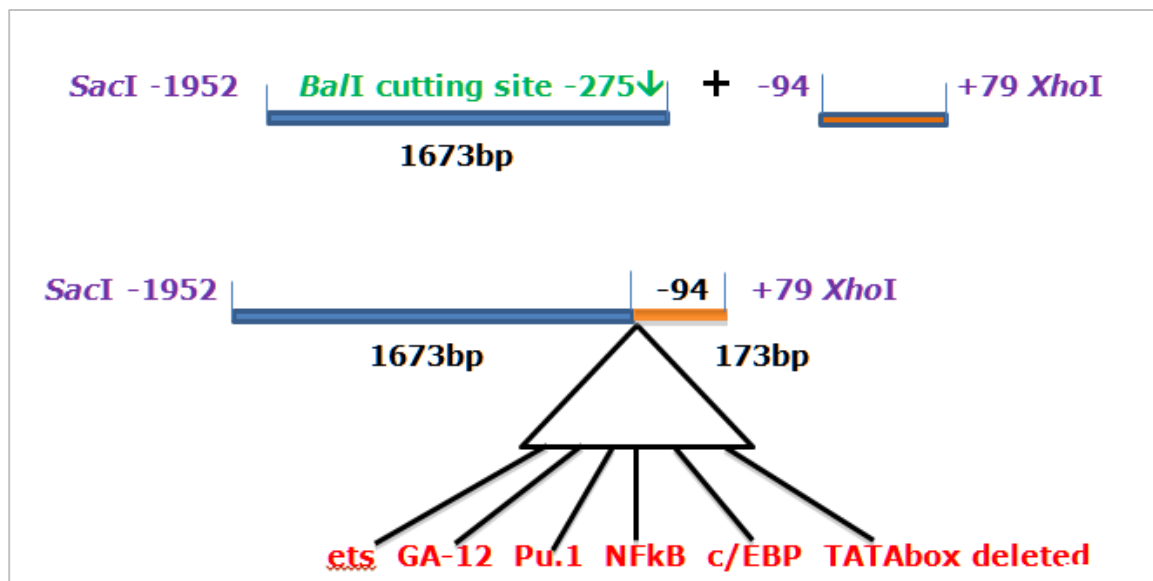


Figure 4.13: Schematic representation of the generation of the deletion construct through ligation of 1673bp fragment (created by digestion of full length promoter by *Ba*I TGG▼CCA at nt-275) to 173bp fragment (created by PCR). Deletion construct of 1848bp in length is shown with transcription factors TATAbox, c/EBP, NFkB, Pu.1, GA-12 and ets deleted.

The primers used for generation of full length fragments (to be digested with *Ba*I) and 173bp fragment are shown in tables 4.3 and 4.4.

Primer	Sequences with 9bp 5' extensions	Incorporated restriction site
Adapted P1F	ACA <u>GAGCTC</u> CTTTTGCATAACTGGC	GAGCT'C <i>Sac</i> I
Adapted P8R	ATA <u>CTCGAG</u> GGAAGTGCTTACCTTGCT	C'TCGAG <i>Xho</i> I

Table 4.3: Primers used for generation of full length promoter - 1952 to +79 (to be digested with *Ba*I, creating 1673bp fragment of deletion construct)

Primer	Sequence with 9bp 5' extension and new forward primer	Incorporated restriction site
New F	GAGAGTTGTTTTCAATGTTGCAACAAGTC	
Adapted P8R	ATA <u>CTCGAG</u> GGAAGTGCTTACCTTGCT	C'TCGAG <i>Xho</i> I

Table 4.4: Primers used for generation of the -94 to +79 region creating 173bp fragment of the deletion construct

Several tubes of each PCR preparation were pooled, electrophoresed on agarose, excised and extracted to ensure that no original template was present. The eluate of the full length (2031bp) fragment was digested with *Ba*I, generating two fragments of 1673bp and 358bp with blunt ends. The digest was electrophoresed on agarose and the larger 1673bp fragment of the digest excised, extracted and then digested with *Sac*I, to create the cloning site at the 5' end. The digested product was cleaned on a spin column. The 173bp fragment was digested with *Xho*I, to create the cutting site at the 3' end and cleaned on a spin column.

On this occasion PCR amplicons were not treated with T4 DNA polymerase to remove the 3' A addition by *Taq* polymerase.

An ethidium bromide stained agarose gel of the PCR products of the full length (2031bp) and 173bp fragments are shown in figures 4.14 and 4.15.

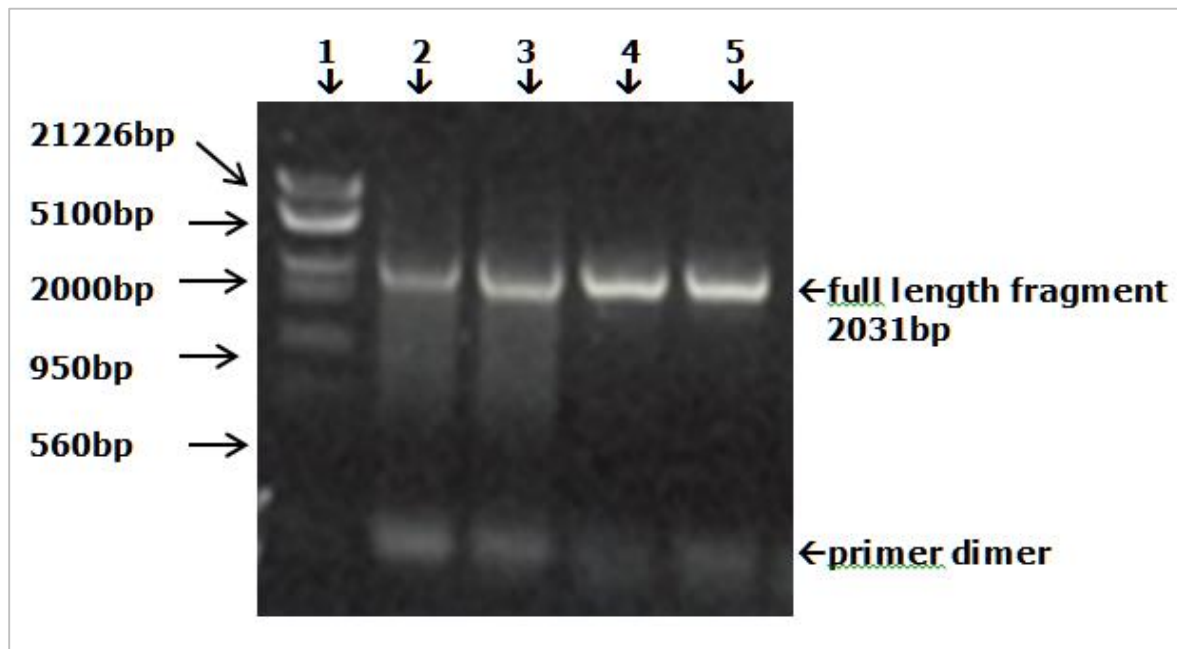


Figure 4.14: Lane 1, λ H/E markers (21226bp-560bp); lanes 2-5, full length fragment (2031bp) (to be digested with *Ba*I) from 4 PCR reactions.

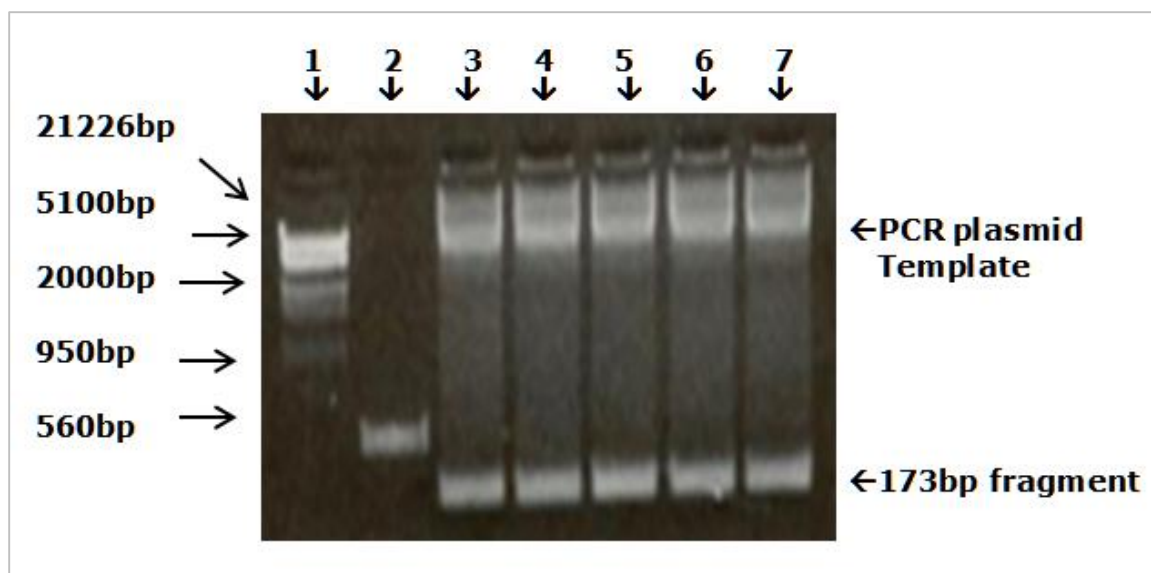


Figure 4.15: Lane 1, λ H/E markers (21226bp-560bp); lane 2, marker (339bp); lanes 3-7, the 173bp fragment (for creation of deletion construct) from 5 PCR reactions.

4.3 Cloning into pGL3-Basic expression vector

As preparation for the reporter assay, the five promoter fragments normal, SNP -1523 G, SNP -1564T (2022bp), truncated (1744bp) and deletion (1848bp) were sub-cloned into the pGL3-Basic expression vector shown in figure 4.16.

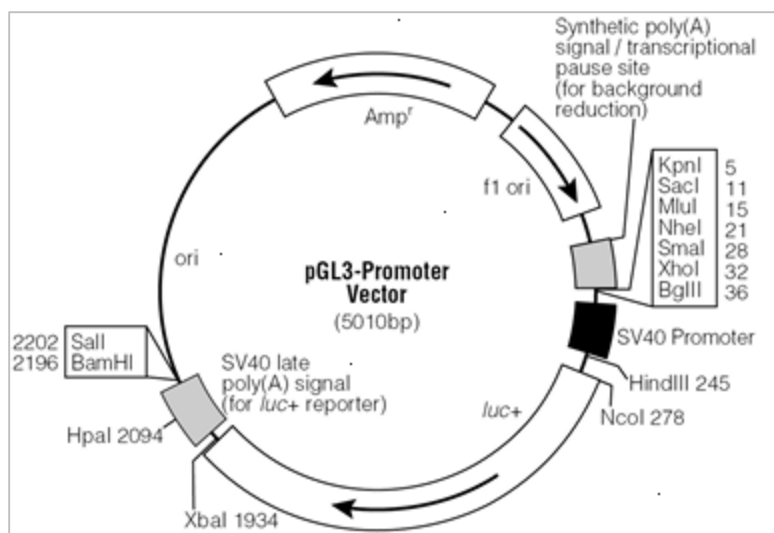


Figure 4.16: pGL3-Basic expression vector circle map and multiple cloning site.

4.3.1 Cloning of normal and SNP -1564 T fragments into pGL3-Basic expression vector

The pGL3-Basic expression vector was linearized by digestion with *SacI* and *XhoI* restriction enzymes and then cleaned on a spin column.

The digested normal and SNP -1564 T inserts were subcloned into the linearized, cleaned pGL3-Basic expression vector by ligation and transformation of competent cells. Master plates of white colonies were prepared and colonies checked for inserts by PCR. Overnight cultures of colonies with confirmed inserts of the correct size were grown for storage at -80°C and plasmid DNA extractions done, as previously described, using the pGEM®-T vector system.

4.3.2 Ligation of fragments for SNP -1523 G fragment and cloning into pGL3-Basic expression vector

When the generation of the correct SNP -1523 G fragment was completed, the pGL3-Basic expression vector had been obtained, so

this fragment was ligated and cloned directly into the expression vector in a single step. Prior to this step, each fragment was digested with *Sac*I and *Xho*I respectively to remove the added overhangs, creating the complementary cloning site for ligation into the expression vector.

An overnight culture of the SNP -1523 G clone was grown for storage at -80°C, plasmid DNA preparation and sequencing to confirm the correct construct sequence. As clones AG4 and AG44 had been previously sequenced, only the sequence of the area of ligation was checked. Sequence traces of the SNP -1523 G and ligation point of the 663bp and 1359bp fragments are shown in figures 4.17 and 4.18. These are depicted separately due to the 236bp distance between

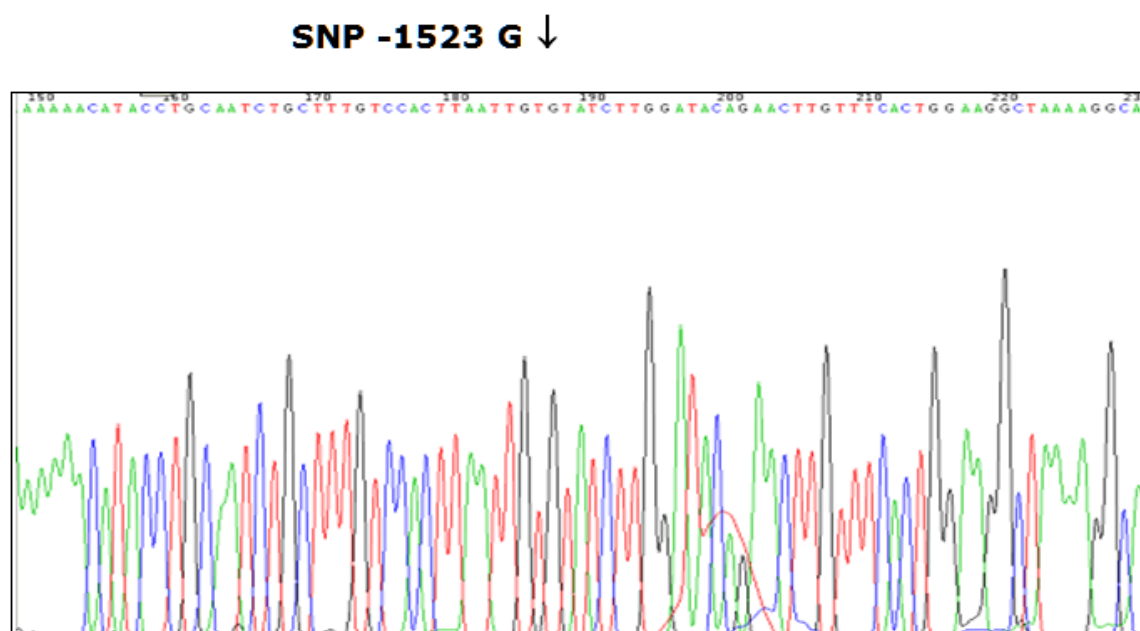


Figure 4.17: A section of the sequence trace of cloned SNP - 1523 G fragment.

Ligation point at *Sma*I CCC↓GGG cutting site

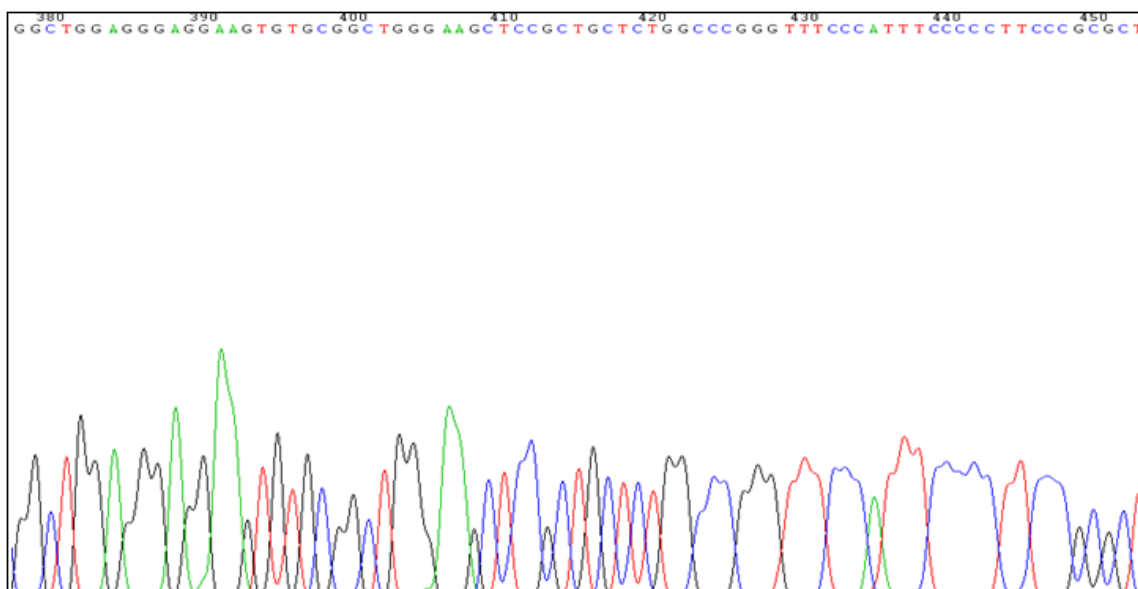


Figure 4.18: Sequence trace of cloned SNP -1523 G fragment showing ligation point of the 663bp and 1359bp fragments at *Sma*I cutting site CCC↓GGG.

The NCBI blast alignment of the cloned SNP -1523 G and normal sequences is shown in figure 4.19.



Figure 4.19: Comparison of cloned SNP -1523 G fragment and normal sequences on NCBI blast alignment search tool, showing correct sequence across region of ligation of 663bp and 1359bp fragments. Boxes show SNP -1523 G and ligation point at *Sma*I cutting site CCC↓GGG. Boxes in this figure correspond with arrows in figures 4.17. and 4.18 above.

4.3.3 Cloning of truncated promoter into pGL3-Basic expression vector

The truncated promoter construct was also cloned directly into the pGL3-Basic vector. Several tubes of PCR product of amplified truncated promoter were prepared, digested with *Sac*I and *Xho*I, creating the cloning site for ligation, cleaned on a spin column and cloned as previously described. Sequencing was done to verify that the fragment was correct. Sequencing trace and NCBI blast alignment are shown in figures 4.20 and 4.21.

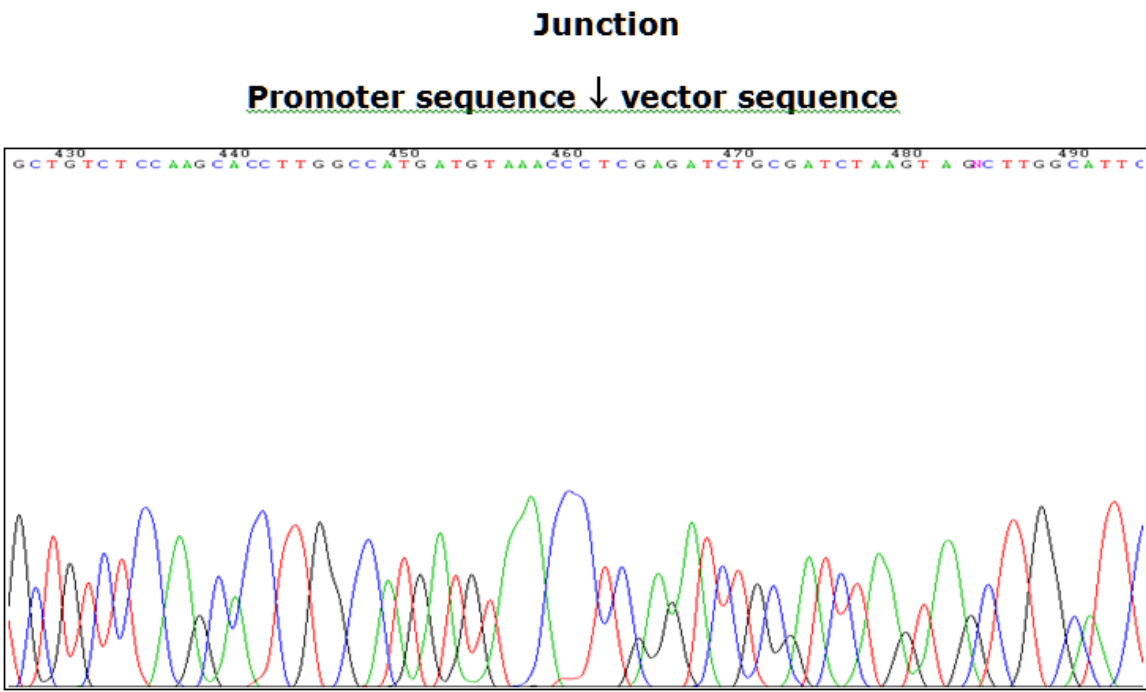


Figure 4.20: Sequencing trace of cloned truncated promoter, showing truncation of sequence at promoter-vector junction.

Query	61	AGTCTTATTAGTGGTACACGCAGTTTATCATCTCCCAGGTCTGTGTCTGTATGAAATG	120
Sbjct	12469	AGTCTTATTAGTGGTACACGCAGTTTATCATCTCCCAGGTCTGTGTCTGTATGAAATG	12528
Query	121	TGCATGGGTGTGTGTGTGCACGCGTGTGTTCCCACTCGGGGAATGTGGGAGAGGTGCAT	180
Sbjct	12529	TGCATGGGTGTGTGTGTGCACGCGTGTGTTCCCACTCGGGGAATGTGGGAGAGGTGCAT	12588
Query	181	GGAGCCAAGATGGGTGGTAAATAGTATGTTTCTGAAATTAAAGGACTAATGTGGAGGAAG	240
Sbjct	12589	GGAGCCAAGATGGGTGGTAAATAGTATGTTTCTGAAATTAAAGGACTAATGTGGAGGAAG	12648
Query	241	GCGCCCCAGATGTACTAAACCCCTTGGCCCTTCATCTCATCCTCTCTGACTTGGGAAGAAC	300
Sbjct	12649	GCGCCCCAGATGTACTAAACCCCTTGGCCCTTCATCTCATCCTCTCTGACTTGGGAAGAAC	12708
Query	301	CAGGATTTTGTGTTTAAAGCCCTTGGGCATACAGTTGTTCCATCCCGACATGAACTCAGCC	360
Sbjct	12709	CAGGATTTTGTGTTTAAAGCCCTTGGGCATACAGTTGTTCCATCCCGACATGAACTCAGCC	12768
Query	361	TCCCGTCTGACCGCCCTTGGCCCTTCCTTCTCCTCGATCTGTGGAACCCAGGGAATCTG	420
Sbjct	12769	TCCCGTCTGACCGCCCTTGGCCCTTCCTTCTCCTCGATCTGTGGAACCCAGGGAATCTG	12828
Query	421	CCTAGTGCTGTCTCCAAGCACCTTGGCCATGATGTAACCC	461
Sbjct	12829	CCTAGTGCTGTCTCCAAGCACCTTGGCCATGATGTAACCC	12869

Figure 4.21: Comparison of cloned truncated promoter and normal sequences on NCBI blast alignment search tool, showing end truncated sequence. Box shows termination point

of promoter sequence. Box in this figure corresponds with arrow in figure 4.20 above.

4.3.4 Ligation of fragments for creation of deletion construct and cloning into pGL3-Basic expression vector

The 1675bp and 173bp were also ligated together in a single step and cloned into the pGL3-Basic expression vector as previously described. Overnight cultures were grown and plasmid DNA extractions prepared on clones with confirmed inserts. Sequencing was done to verify that the sequence spanning the deletion/ligation site was correct. Sequence trace and blast alignments are shown in figures 4.22 and 4.23.

Ligation point of 2 fragments creating deletion

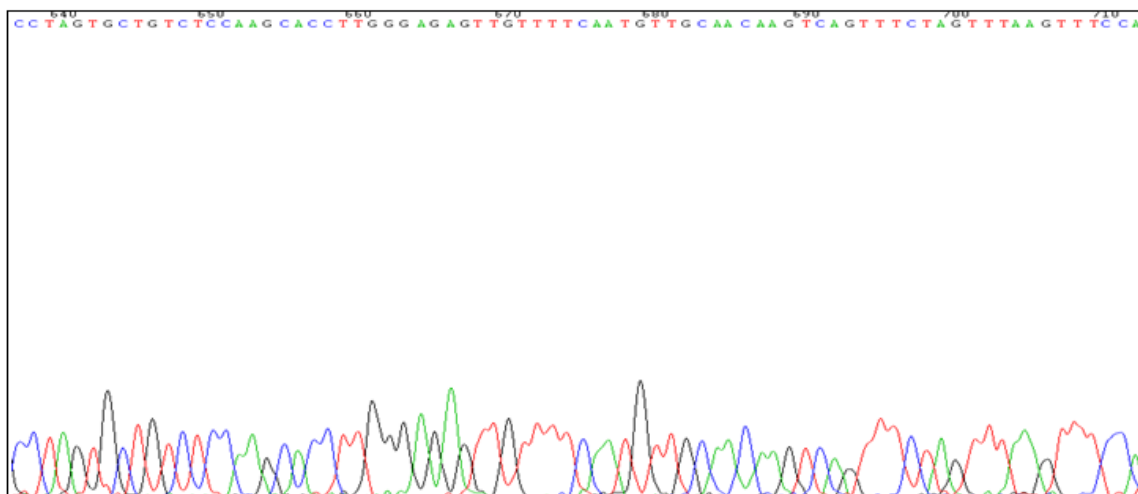


Figure 4.22: Sequence trace showing ligation point of 1675bp and 173bp. fragments at *Ba*/I cutting site TGG↓CCA, creating deletion.

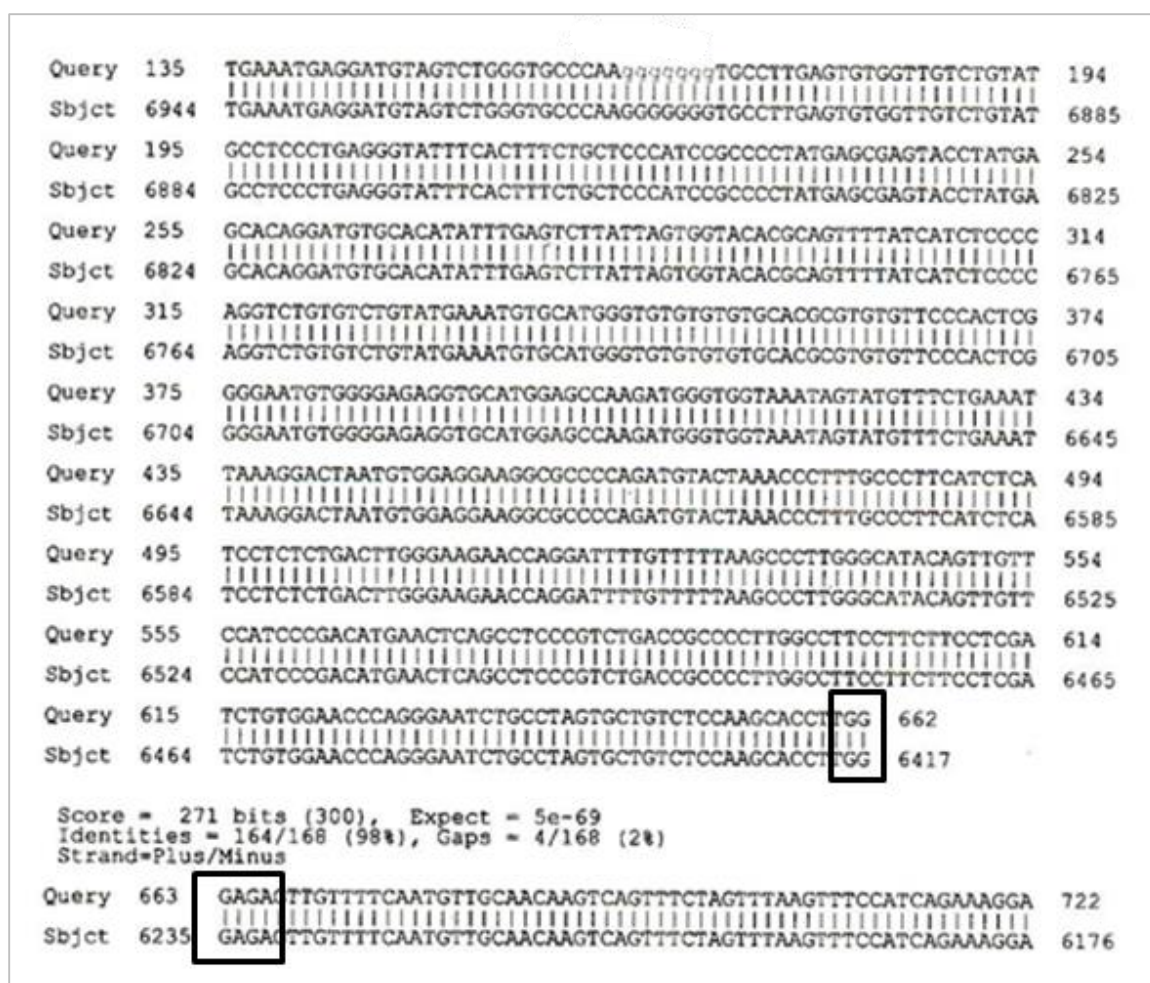


Figure 4.23: Comparison of cloned deletion promoter and normal sequences on NCBI blast alignment search tool, showing ligation point of the 1673bp and 173bp fragments. Comparison alignments are shown in two sections due to the deleted area between them, where the normal sequence did not align. Boxes show the *Ba*/I cutting site TGG↓CCA in larger 1675bp fragment and correct sequence after ligation to smaller 173bp fragment. Boxes in this figure correspond to arrow in figure 4.22 above, showing correct ligation of two fragments.

The normal, SNP -1523 G and SNP -1564 T IL12p40 promoter fragments were successfully cloned into the pGL3-Basic expression

vector. A truncated IL12p40 promoter of 1744bp and one of 1848bp with essential elements deleted were created and cloned into the pGL3-Basic expression vector, providing two negative control constructs for the reporter assay.

Plasmid DNA extractions of five clones containing each of the five fragments were prepared from overnight cultures (20ml of each) and checked by visualization on an agarose gel, and concentrations measured, for the reporter assay. The concentrations of the plasmid DNA preparations were: normal fragment 296ng/ μ l, SNP -1523 G fragment 343.6 ng/ μ l, SNP -1564 T fragment 335.3ng/ μ l, truncated promoter fragment 422.2ng/ μ l, promoter fragment with deletion 310.1ng/ μ l. This provided sufficient plasmid DNA for all transfection experiments.

Plasmid DNA minipreps of clones with the five promoter inserts (normal, SNP -1523 G, SNP -1564 T, truncated and deletion constructs) in the pGL3-Basic expression vector are shown in figure 4.24. The size of the vector (4818bp) prevents the size differences of the fragments to be seen on this agarose gel.

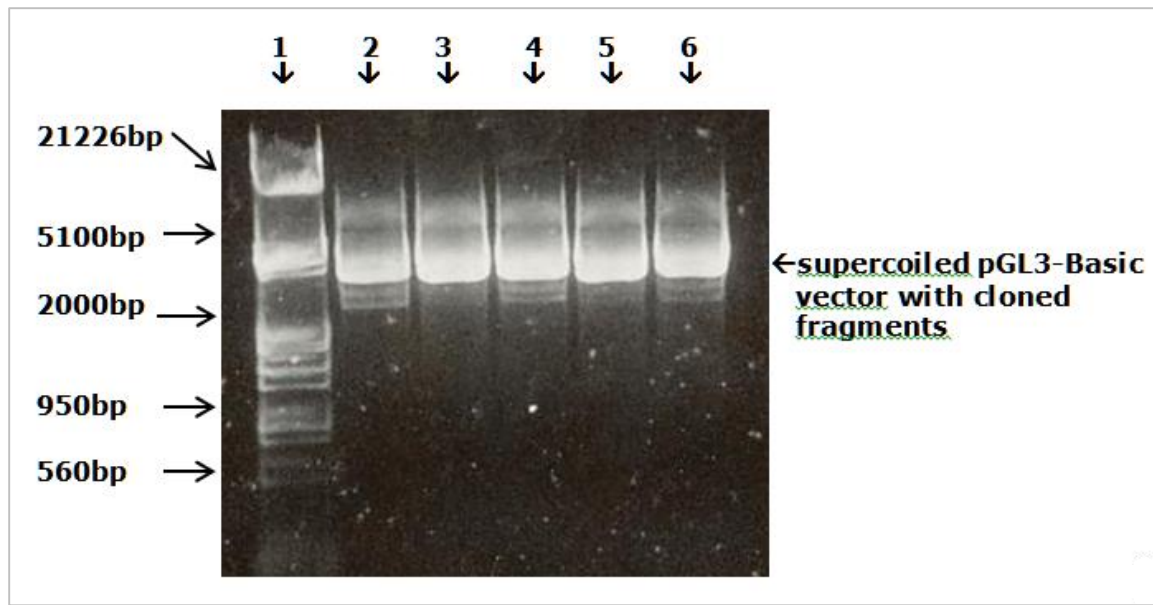


Figure 4.24: Agarose gel (1%) of plasmid DNA minipreparations of the 5 cloned promoter fragments in the pGL3-Basic expression vector. Lane 1, λ H/E markers (21226bp-560bp); lane 2, normal promoter; lane 3, SNP -1523 G; lane 4, SNP -1564 T; lane 5, truncated promoter; lane 6, promoter with deletion.

4.4 Transfection experiments

4.4.1 Background

Transfection is the delivery of DNA, RNA, proteins or other macromolecules into the interior of cultured cells. Without intervention, mammalian cells take up and express externally applied DNA with very low efficiency, due to the lipid bilayer of the eukaryotic cell membrane which acts as a barrier. Transfection methods increase the efficiency of uptake and are based largely on three different strategies that rely on 1) carrier molecules, 2) liposomes and 3) encapsulation with viral proteins (Sambrook, Fritsch and Maniatis 1989). In the case of nucleic acid transfection, carrier molecules

utilize the fact that negatively charged nucleic acids can readily adsorb to positively charged carrier molecules through electrostatic interaction. The carrier/nucleic acid complex is taken up by the cells and the nucleic acid is released within the cytoplasm or nucleus of the cell. The first method that used this principle was the calcium phosphate or DEAE-dextran method, described by Graham and van der Eb in 1973. This method is pH dependent and can be toxic to cells, so today transfection reagents usually belong to the lipid/liposome group (Current Protocols in Molecular Biology Volume 1 [Eds Asubel *et al*] John Wiley and sons 1989).

Cationic liposomal reagents have improved DNA transfection methods. If a cationic lipid is mixed with a neutral lipid, unilamellar liposome vesicles are formed, carrying a net positive charge. Nucleic acids can adsorb to these vesicles, gaining access to the interior of cells, by fusion of the liposome either with the plasma membrane or the membrane of an endocytic vesicle. The next generation of these transfection reagents has been the multicomponent reagents consisting of lipids and proprietary components. This new type of transfection reagent exhibits lower cytotoxicity and more transfection efficiency.

More forceful, direct methods of delivery include electroporation, which generates short lived pores in the cell membrane and bombardment with nucleic acid coated carrier particles by *eg* a gene gun and microinjection. These methods may be used when working with plant cells, bacteria or transgenic mice, but are not generally suitable for transfection of cultured mammalian cells.

Nucleic acid transfection can be done by using viral vectors such as recombinant vaccinia viruses, retroviruses and adenoviruses.

Transfected nucleic acids can be transiently expressed, permanently expressed or not expressed at all. DNA that has crossed the membrane barrier will be transported to the nucleus of the eukaryotic cell, where it will be transcribed if it contains a suitable promoter. Expression usually ceases after two to four days since episomal DNA is degraded by the cells (Sambrook, Fritsch and Maniatis 1989). Permanently expressing cell lines can be generated by incorporating the foreign DNA/gene into the genome of the transfected cells. This is readily achieved through use of retroviral vectors.

Several factors need to be considered when planning a transfection experiment as results of expression studies may be critically influenced by several factors.

Correct choices of cell line and culture conditions are vitally important. Dividing cells tend to be more accessible for the uptake and expression of foreign DNA compared to quiescent cells. Transfection efficiencies can differ between adherent and suspension cells. Passage number can affect transfection efficiency as cell features may change over time in culture. Other important factors include choice of vector, appropriate transfection reagent, ratio of transfection reagent to DNA in the transfection complex (charge ratio), time allowed for transfection complex to form, volume of transfection complex added, transfection medium and cell density at the time of transfection.

For the work of this thesis, the Dual-Luciferase® Reporter Assay System was used to assess the functional relevance of the SNP -1523 A/G and SNP -1564 C/T heterozygous changes found in the IL12p40 promoter in the subjects screened.

The term “dual reporter” refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. The experimental reporter is correlated with the effect of specific experimental conditions, while the activity of the co-transfected control reporter provides an internal control that serves as the baseline response. Normalizing the activity of the experimental reporter to the activity of the internal control minimizes experimental variability caused by differences in cell viability, transfection efficiency, or lysis efficiency. In this way, dual reporter assays reduce extraneous influences, allowing more reliable interpretation of results.

4.4.2 Methods and results

4.4.2.1 Optimization

In optimizing experiments, three cell lines, two internal controls, three transfection reagents and different DNA:transfection reagent ratios were used. Two incubation times of 34 hours and 48 hours were used.

The mouse macrophage RAW 264.7, human monocyte THP-1 and human fibroblast HT1080 cell lines were used. In the optimization experiments, only the RAW 264.7 cell line showed an increase of fold induction on stimulation with IFN γ and LPS. Very low readings were obtained with the THP-1 and HT1080 cell lines with RLUs measuring <0.001. This is shown in figure 4.25.

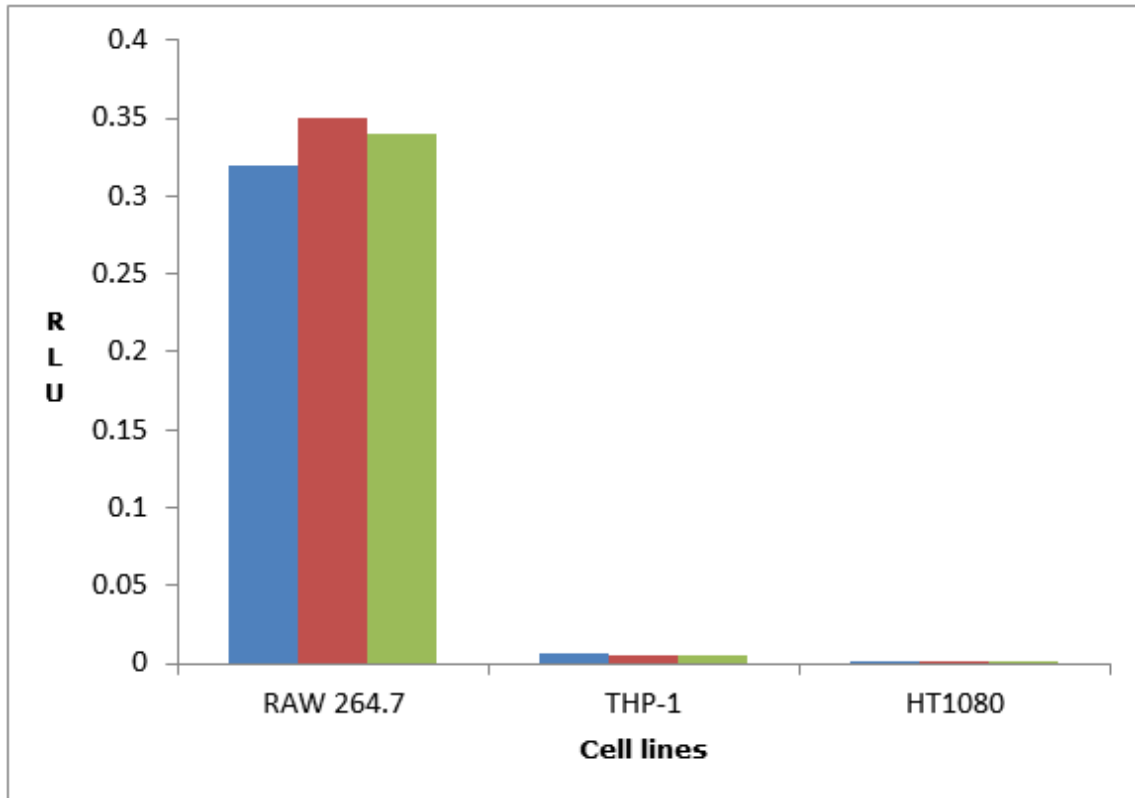


Figure 4.25: RLU readings from triplicate experiments [experiment 1 =blue, experiment 2 = red and experiment 3 = green] using three different cell lines i.e. 1) mouse macrophage RAW 264.7, 2) human monocyte THP-1 and 3) human fibroblast HT1080, transfected with normal L12p40 promoter construct and stimulated with IFN γ (final concentration 1ug/ml) and LPS (final concentration 1ug/ml).

Two concentrations of two internal controls pRL-TK (with thymidine kinase promoter 50ng) and pRL-CMV (with cytomegalovirus promoter 10ng) were used. The pRL-TK was the internal control vector of choice as no advantage was seen with pRL-CMV and there was more stock of the former available. Results are shown in figure 4.26.

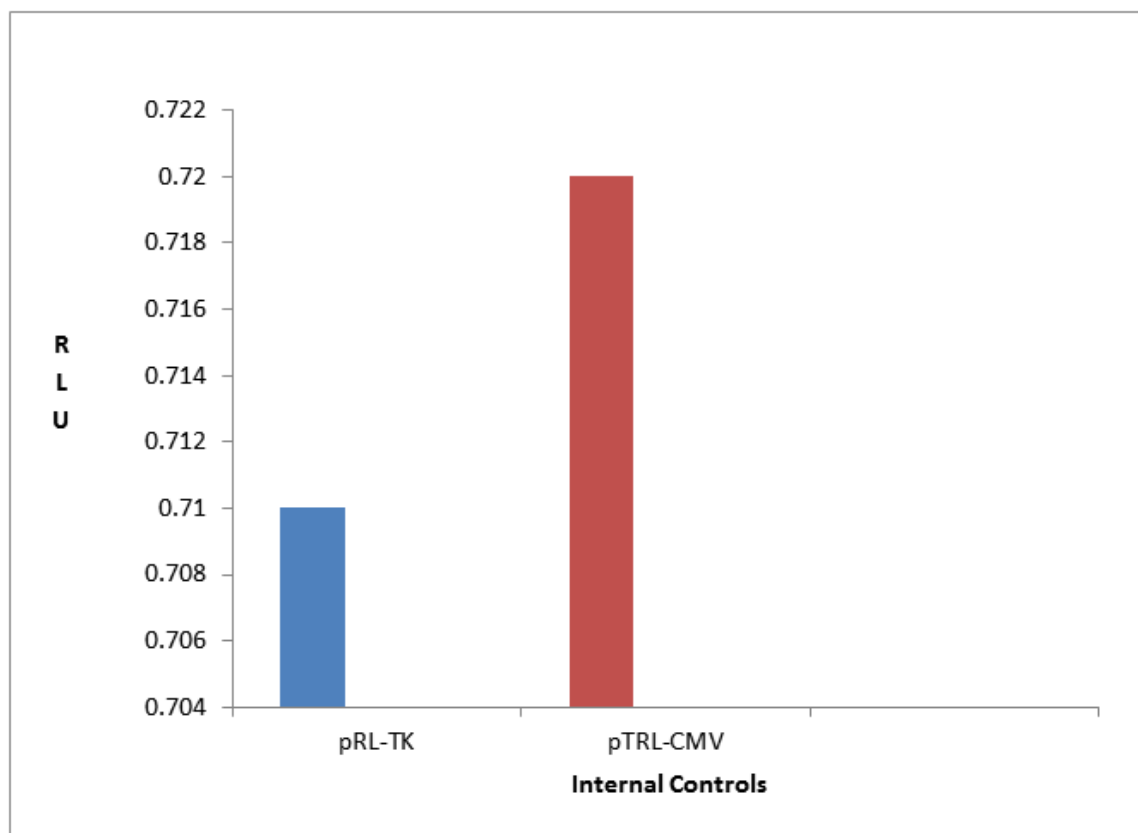


Figure 4.26: RLU readings from RAW 264.7 cells transfected with normal IL12p40 promoter construct using internal controls:

1) pRL-TK [blue] 2) pRL-CMV [red].

The three transfection reagents gave similar results, but X-tremeGene HP DNA Transfection Reagent, the recommended agent for cells known to be difficult to transfect, was used. Results are shown in figure 4.27.

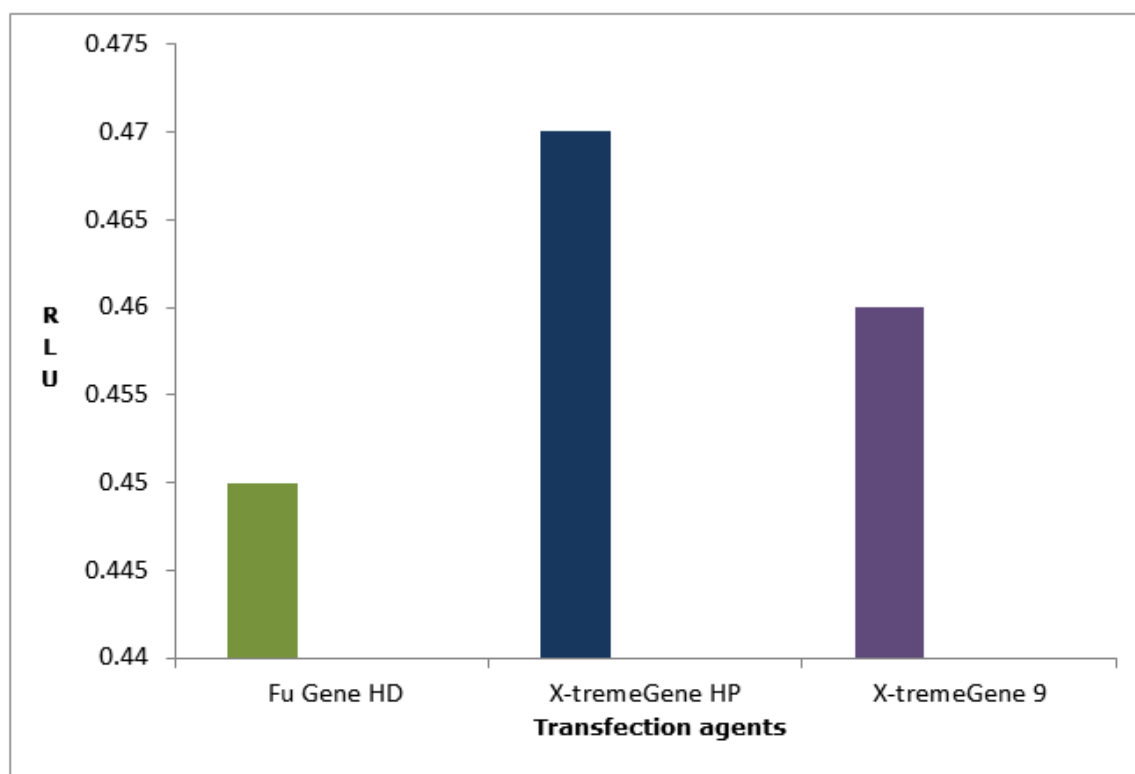


Figure 4.27: RLU readings from RAW 264.7 cells transfected with normal IL12p40 promoter construct using transfection reagents: 1) Fu Gene HD [green] 2) X-tremeGene HP [blue] 3) X-tremeGene 9 [purple].

The recommended incubation time of 48 hours was used, for convenient and timely addition of stimulants, as no advantage was observed with the shorter time of 34 hours.

4.4.2.2 Transfection experiments

A typical transfection experiment took 5 days. The following is a brief summary as detailed methods are described in chapter 6, General Methods.

On day 1, cells were plated into a 12 well tissue culture plate and grown overnight to a confluency of 60-70%.

On days 2 and 3, the DNA mixes of five cloned promoter fragments (normal, SNP -1523 G, SNP -1564 T, truncated, deletion) in pGL3-Basic expression vector and pRL-TK internal control vector were prepared. This was followed by preparation of the transfection reagent and subsequent DNA transfection into the RAW 264.7 cells. The cells were incubated at 37°C in 5% CO₂ for a total of 48 hours, with the addition of DMSO, IFN γ and LPS at specific allotted times according to experimental design. IFN γ primes the IL12p40 gene promoter for response to LPS and DMSO has been reported to enhance the effect (Ma *et al.* 1996, Gri *et al.* 1998, Becker *et al.* 2001).

On day 4 the cells were lysed, harvested and frozen overnight at -80°C.

On day 5, the Relative Light Units (RLUs) were determined by luminometry

A transfection control (transfection control construct was kindly donated by the South African Aids Vaccine Institute laboratory, Institute of Infectious Disease and Molecular Medicine, UCT) was included in each experiment by transfecting the RAW 264.7 cells with the normal promoter fragment together with the control construct LucZ/SV40 in the pGL4-Basic expression vector. This control construct, containing the strong SV40 promoter, was a measure of transfection efficiency. It gave similar or higher readings than the experimental transfection constructs on the luminometer.

4.4.2.3 Transfection Results

Test results were normalized as follows: background readings (empty vector and/or unstimulated) were subtracted from all readings. The firefly readings were normalized against the internal control pRL-TK

readings which were taken as 1. The fold induction values for the normal, SNP -1523 G, SNP -1564 T and truncated promoter were calculated as normalized firefly value/normalized deletion promoter. No fold induction was observed for the truncated promoter construct, p values were not significant. ($p = 0.49$ G vs T; $p = 0.74$ Normal vs G; $p = 0.35$ Normal vs T) (p value is for Kruskal Wallis one-way analysis of variance).

The relative luciferase activity (fold induction above deletion promoter construct) of the stimulated RAW 264.7 cells transfected with the normal, SNP -1523 G and -1564 T promoter constructs is shown in figure 4.28.

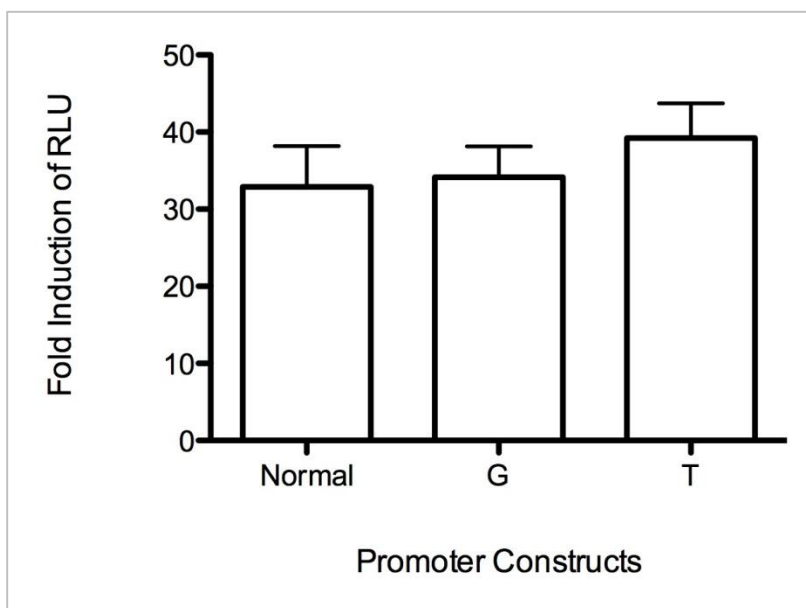


Figure 4.28: Relative luciferase activity in RAW264.7 cells transfected with normal, SNP -1523 G and SNP -1564 T promoter constructs in pGL3-Basic expression vector and stimulated with DMSO (1.2%), IFN γ (1 μ g/ml) and LPS (1 μ g/ml).

4.5 Conclusions

The functional effect of any mutation, particularly a heterozygous mutation where the effect is diluted, may be subtle in a clinical condition.

In a reporter assay, effects of single alleles are tested. In this series of experiments, a functional effect of the promoter SNPs was not demonstrated and no gross differences were observed between the normal and SNP -1523 G or SNP -1564 T readings.

Unless the functional effect is marked, a reporter assay of this kind may not be sensitive enough to show subtle functional differences.

To further evaluate whether the SNPs had any effect on the immunological phenotype of paediatric patients and controls measurement of functional phenotype was done by measurement of upregulated cytokines in WBAs. This is described in chapter 5.

CHAPTER 5: Immune Testing

5.1 Introduction

Cytokines are humoral proteins or glycoproteins with immunomodulatory functions. The clinical outcome of many infections appears to be influenced by the overall balance of production of pro-inflammatory and anti-inflammatory cytokines (Hassig *et al.* 1998, Bidwell *et al.* 1999a).

Cytokines modulate the activities of target cells, binding to specific cytokine receptor ligands, resulting in gene activation. Cytokine production by cells of the immune system may occur through non-antigen specific and antigen specific stimuli. An example of a non-antigen specific response would be when monocytes are exposed to bacterial cell wall products such as LPS, resulting in production of IL12 and other cytokines. Antigen specific responses are generated by B and T cells through immunoglobulin and T cell receptors respectively.

Cytokine and cytokine receptor genes are generally highly conserved in terms of exon sequences, but even silent mutations may influence protein expression by altering mRNA splicing, mRNA stability and gene transcription. A single base change can increase or decrease the binding of a transcription factor. Polymorphisms within the 5' to 3' regulatory sequences or introns of genes may have a significant effect on transcription. In several cytokine genes, polymorphisms within promoter regions, mostly single nucleotide polymorphisms (SNPs) or microsatellites which affect gene transcriptions causing inter individual variations in cytokine production have been described (Bidwell *et al.* 1999a, Bidwell *et al.* 1999b).

Many of the reported polymorphisms within cytokine genes occur within known or putative regulatory regions (Bidwell *et al.* 1999a, Bidwell *et al.* 2001). These changes may have a significant effect on transcription, since they may alter the structure of transcription factor binding sites within gene promoters, or the structure of enhancers and silencers within introns or at more remote regulatory sites.

In the first early studies, cytokine gene polymorphisms were shown to be involved in the susceptibility, severity and clinical outcome of several infectious diseases (Bidwell *et al.* 1999a, Bidwell *et al.* 2001, Hill 1998) and may play a role in mortality in children and the elderly (Pawelec *et al.* 1999).

The influence of cytokine gene polymorphisms on gene expression and disease has been addressed by two approaches in research, namely, studies using *in vitro* gene expression and studies looking at *in vivo* disease association.

The *in vitro* gene expression studies examine the relationship between individual polymorphic alleles of cytokine genes and the expression of the transcript or cytokine *in vitro*. The approach has usually included measuring the level of cytokine or cytokine receptor mRNA, or of cytokine or receptor protein, expressed as a result of *in vitro* stimulation of cells in culture with a mitogen and isolation of individual alleles of gene promoters by cloning them adjacent to a reporter gene in an expression vector.

The *in vivo* association studies have attempted to identify genetic markers for a given disease. The genetic analysis of cytokines in human disease has focused on case-control association studies in which the frequencies of marker alleles in groups of patients and

healthy controls are compared and the difference is subjected to statistical analysis. One of the limitations of this approach is the difficulty in identifying a perfectly matched control group.

5.2 Immune phenotype

An immune phenotype which is defined by cytokine and cytokine receptor measurements, can predict genetic defects. Following is a description of some phenotypes that present with genetic defects within and outside of the IL12/IL23/IFN γ pathway, shown in table 5.1.

Deficiency	Immune phenotype	Reference
IFNγR1		
Complete recessive	No response to IFN γ , ratio of TNF α production in response to LPS+IFN γ /LPS low. Reduced in vivo production of IFN γ , IL12 and TNF α . High levels of plasma IFN γ . Absent receptor expression.	Newport <i>et al.</i> 1995, Holland 2001, Fieschi <i>et al.</i> 2001
Partial recessive	Diminished response to IFN γ . Response increased by using high concentrations of IFN γ . Expression of non-functional receptor.	Jouanguy <i>et al.</i> 1997b
Partial dominant	Diminished response to IFN γ . Response increased by using high concentrations of IFN γ . Over-expression of non-functional receptor.	Jouanguy <i>et al.</i> 1999, Villela <i>et al.</i> 2002
IFNγR2		
Complete autosomal recessive	No response to IFN γ , ratio of TNF α production in response to LPS+IFN γ /LPS low. High levels of plasma IFN γ . Absent receptor expression	Dorman and Holland 1998, Jouanguy <i>et al.</i> 2000, Fieschi <i>et al.</i> 2001

Partial autosomal recessive	Diminished response to IFN γ . Response increased by using high concentrations of IFN γ . Reduced expression of non-functional protein.	Doffinger <i>et al.</i> 2000a, Doffinger <i>et al.</i> 2001.
IFNγ		
Acquired autoantibodies	Undetectable IFN γ in whole blood. In absence of autologous plasma, detectable IFN γ levels secreted by PBMCs.	Kampmann <i>et al.</i> 2005
IL12- and IL23Rβ1		
Complete autosomal recessive	No response to IL12. IFN γ production impaired. Absent receptor expression.	Sakai <i>et al.</i> 2000, Lammas <i>et al.</i> 2001
Partial autosomal recessive	Diminished response to IL12. Internal receptor measured.	Lichtenauer-Kaligis <i>et al.</i> 2003
IL12p40		
Complete autosomal recessive	Undetectable or low IL12. Normal receptor expression.	Altare <i>et al.</i> 1998c
STAT1		
Complete autosomal recessive	Undetectable or low IL12, TNF α , IFN γ levels.	Doughty <i>et al.</i> 2001, Gautier <i>et al.</i> 2005.
Partial autosomal recessive	Low IFN γ levels, undetectable IL12p40 production.	Chapgier <i>et al.</i> 2006b
NEMO		
Partial X-linked recessive	Undetectable or low IFN γ and IL12 levels.	Filipe-Santos <i>et al.</i> 2006
CYBB		
Partial X-linked	Impaired CYBB dependent NADPH respiratory burst.	Bustamante <i>et al.</i> 2011

recessive		
IRF-8		
Partial autosomal dominant	Impaired IL12 production.	Hambleton <i>et al.</i> 2011, Salem <i>et al.</i> 2013
Complete autosomal recessive	Impaired IL12 production.	Hambleton <i>et al.</i> 2011, Salem <i>et al.</i> 2013
ISG15		
Complete autosomal recessive	Low IFN γ levels produced by PBMCs and NK.	Bogunovic <i>et al.</i> 2012

Table 5.1: Immune phenotypes of deficiencies causing MSMD

5.3 Immunological Investigations

Cytokine assays can be used to probe the site of defects in patients with single gene mutations in the IL12/IL23/IFN γ pathway (Lammas *et al.* 2000).

Cytokines do not function in isolation, but operate within a complex network. IL12 plays a pivotal role in cell mediated immunity, enhancing the cytotoxic activity of NK and T cells and stimulating IFN γ production. The effects and responses of TNF α , IL12 and IFN γ all interact within this cytokine network.

Immunological investigations for genetic defects include whole blood assays followed by ELISA for the measurement several cytokines, including TNF α , IL12p70, IFN γ and flow cytometry for measurement of the cytokine receptors.

Known primary and secondary immunodeficiencies, including HIV infection need to be excluded before investigating patients for possible genetic defects.

5.3.1 $\text{TNF}\alpha$

For $\text{TNF}\alpha$ measurement, whole blood is stimulated with LPS and LPS + $\text{IFN}\gamma$. The ratio of $\text{TNF}\alpha$ levels secreted in response to LPS + $\text{IFN}\gamma$ /LPS is a reflection of the ability of cells to respond to $\text{IFN}\gamma$. Typically a patient with an $\text{IFN}\gamma\text{R}$ defect that abolished function, preventing binding of the ligand to its receptor, would have a low ratio, close to 1.

5.3.2 IL12p70

For IL12 (IL12p70) measurement, whole blood is stimulated with LPS+ $\text{IFN}\gamma$ as $\text{IFN}\gamma$ augments production of IL12. The IL12 levels secreted in response to this stimulation are a reflection of the ability of cells in the blood to produce IL12.

5.3.3 $\text{IFN}\gamma$

For $\text{IFN}\gamma$ measurement, whole blood is stimulated with PHA, a nonspecific T cell stimulant, PHA+IL12 and PPD.

PHA is a non-specific T cell mitogen that cross links T cell receptors. It is commonly used as a positive control to measure the capacity of cells to produce $\text{IFN}\gamma$ in general, rather than to a specific stimulus.

As IL12 is important for optimal $\text{IFN}\gamma$ production, the additive effect of exogenous IL12 on $\text{IFN}\gamma$ responses to PHA is used as an indicator of functional response to IL12 (Altare *et al.* 1998d). The ratio of $\text{IFN}\gamma$ levels produced in response to PHA+IL12/PHA can be used as an indicator of the ability to respond to IL12. Typically a patient with an IL12R defect that affected function, preventing binding of the ligand to the receptor, would have a low ratio.

The $\text{IFN}\gamma$ levels secreted in response to PPD, an extract of *M. tb*, indicate a memory response to mycobacterial antigens. Thus, children

who have been infected with *M. tb*, would be expected to produce $\text{IFN}\gamma$ in response to PPD irrespective of whether they have clinical or latent disease.

Details of assays for probing defects in cytokine production described above are shown in figures 5.1 and 5.2.

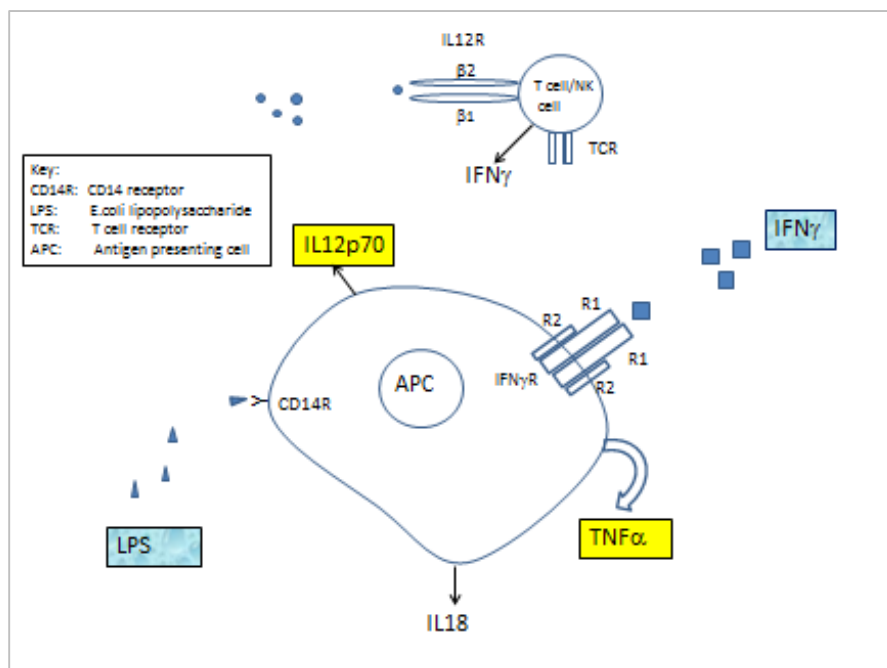


Figure 5.1: Diagrammatic representation of the diluted WBA for measuring $\text{TNF}\alpha$ and IL12p70.

E. coli LPS binds to the CD14 receptor on the antigen presenting cell/monocyte and stimulates $\text{TNF}\alpha$ production. Pre-incubation with $\text{IFN}\gamma$ enhances production in the presence of functional $\text{IFN}\gamma\text{R}$. IL12p70 is produced by the activated monocyte. Yellow boxes indicate cytokines measured ($\text{TNF}\alpha$ and IL12p70) in assay supernatant and turquoise boxes indicate stimulatory reagents (LPS and $\text{IFN}\gamma$) added in the WBA.

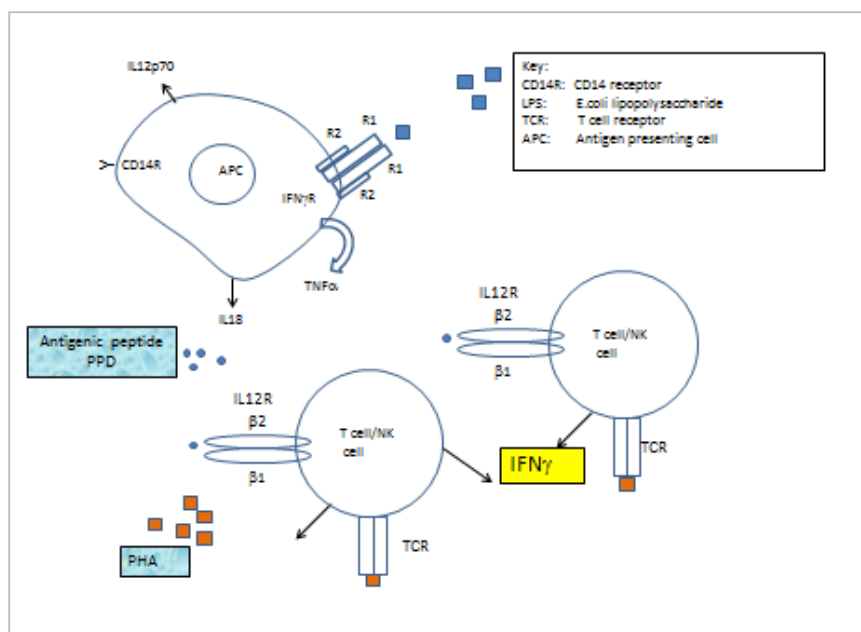


Figure 5.2: Diagrammatic representation of the diluted WBA for measuring IFN γ .

Mycobacterial antigen is processed by the macrophage and presented, with MHC Class II receptor, to the T cell that produces IFN γ . Addition of IL12p70 enhances IFN γ production if IL12R β 1 receptors are functional. The yellow box indicates cytokine measured (IFN γ) in assay supernatant and turquoise boxes indicate stimulatory reagents (PHA, IL12 and PPD) added in the WBA.

5.3.4 Cell surface receptors

The surface cell expression of the cytokine receptors are measured by flow cytometry.

5.4 Methods and results

As a screen, a quantitative immune phenotype was measured on TB cases and healthy controls in the paediatric cohort. The screen included measurement of TNF α , IL12p70 and IFN γ cytokines and the expression of the IL12R β 1 receptor.

Following is a summary of the methods, which are fully described in chapter 6, General Methods.

5.4.1 Sample Collection

For cytokine measurement, venous blood was collected into preservative free heparin and whole blood assays set up within a maximum of 4 hours of collection. IL12R β 1 expression was measured on stimulated PBMCs.

5.4.2 WBAs for measurement of TNF α , IL12p70 and IFN γ .

WBAs were done by diluting heparinized blood 1 in 10 in RPMI medium and then stimulating with specific antigens, viz LPS and IFN γ for TNF α and IL12p70; PHA, IL12 and PPD for IFN γ .

These whole blood assays were optimized using small blood volumes and set up on 96 well plates for large scale screening.

To reduce the potential effect of diurnal variation in cytokine response (Petrovsky and Harrison 1997b), patients were always bled in the mornings.

Many factors can influence the expression of cytokine genes, for example the diurnal immune variation (Petrovsky and Harrison 1997b), linkage disequilibrium with MHC alleles (Petrovsky and Harrison 1997a), as well as the age of donors of blood samples, due to age affecting T cell activation (Lio *et al.* 1996, Pawelec *et al.* 1999).

Active TB patients were bled at recruitment and then at 6 and 12 months after commencing TB treatment to ensure that the disease itself was not influencing cytokine responsiveness. Children with a past medical history of TB and healthy controls were bled twice at an interval of 2 to 4 weeks

to ensure that the measured functional phenotype was stable.

5.4.3 ELISAs

Cytokine levels in the harvested supernatants from the WBAs were measured by in house ELISAs optimized for use in the Burroughs-Wellcome funded collaborative study by Dr Suzanne Anderson. Colour development was then read in a Versamax ELISA plate reader. Cytokine concentrations were calculated using ELISA software from a standard curve run in duplicate on every plate.

The standard curves used for the $\text{TNF}\alpha$, IL12p70 and $\text{IFN}\gamma$ ELISAs are shown in figures 5.3, 5.4, 5.5. Samples readings were read from the linear part of the standard curve.

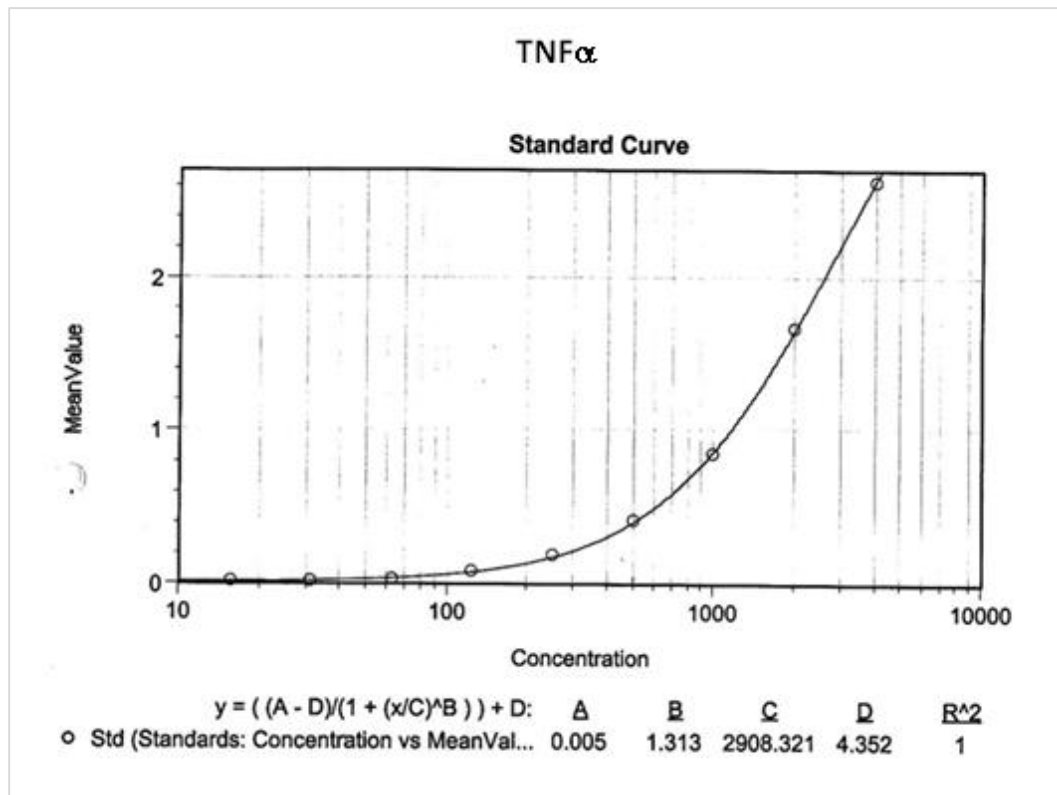


Figure 5.3: $\text{TNF}\alpha$ ELISA standard curve generated by nine standards 30pg/ml – 2000pg/ml.

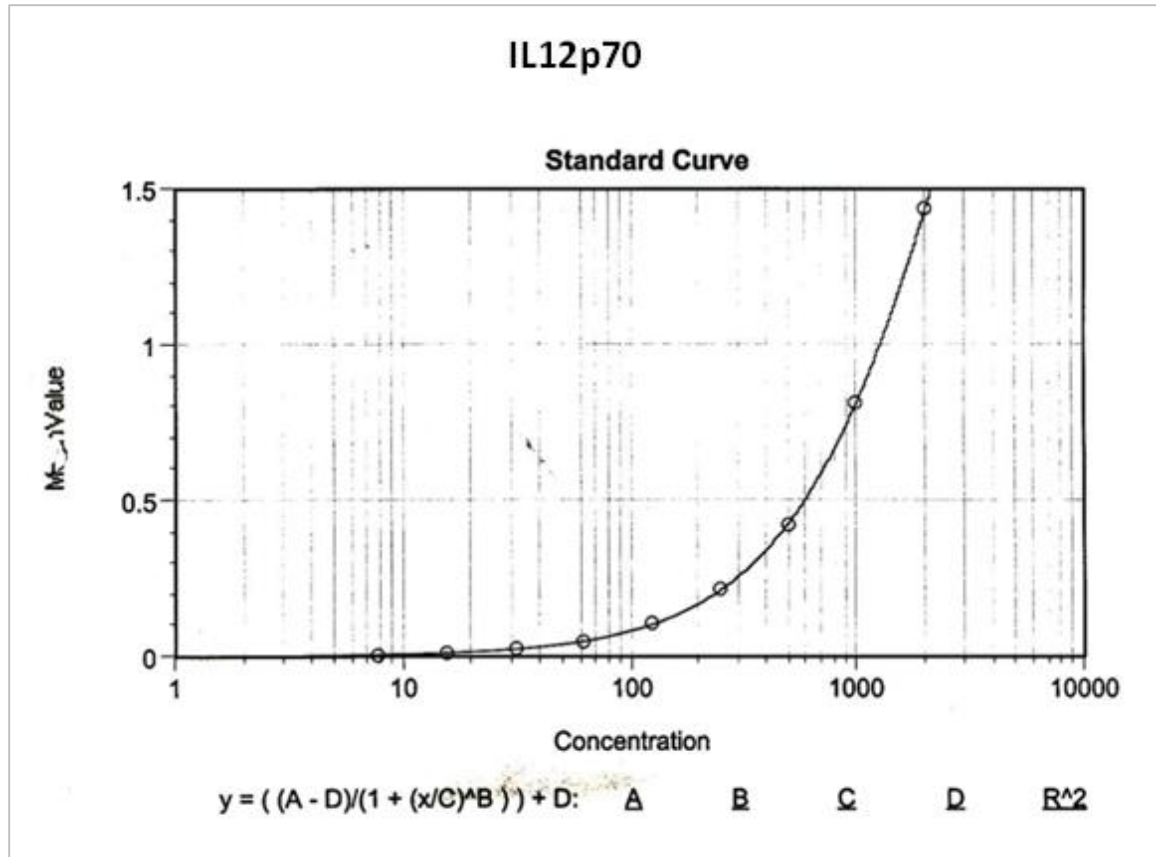


Figure 5.4: IL12p70 ELISA standard curve generated by nine standards 15pg/ml – 2000pg/ml.

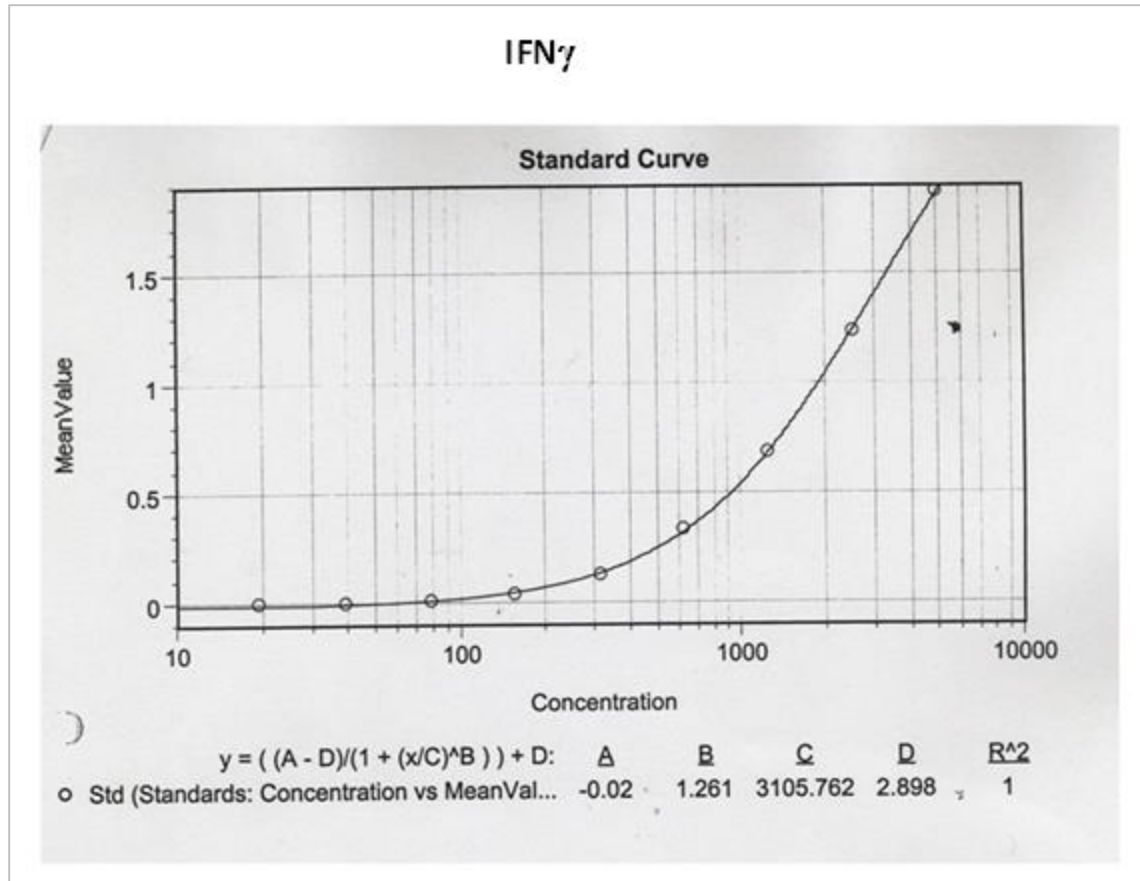


Figure 5.5: IFN γ ELISA standard generated by nine standards 40pg/ml – 5000pg/ml.

The lower and upper detection limits were 30pg/ml to 2000pg/ml for TNF α , 15pg/ml to 2000pg/ml for IL12p40 and 40pg/ml to 5000pg/ml for IFN γ (second lowest to second highest standards on the standard curve).

5.4.3.1 Cytokine measurement

For patients with active TB, bloods for cytokine testing were taken at baseline (pre commencement of TB treatment), 6, 12 and 24 months later.

For evaluation of cytokine production between groups, levels measured in acute cases collected at least 12 months after diagnosis

and commencement of TB treatment were used for comparison with Past History TB cases and healthy controls.

5.4.3.2 $\text{TNF}\alpha$ responses

The ability to produce $\text{TNF}\alpha$ in response to *E. coli* LPS and respond to $\text{IFN}\gamma$ was assessed by measuring the ratio of $\text{TNF}\alpha$ produced to LPS alone and following the addition of exogenous $\text{IFN}\gamma$. This was expressed as a ratio of $\text{TNF}\alpha$ production ($\text{LPS} + \text{IFN}\gamma / \text{LPS}$). Responses of all TB cases, Active and Past History combined, were significantly lower than those of the healthy controls ($p < 0.0001$), shown in figure 5.6 (a).

To evaluate if the lower $\text{TNF}\alpha$ ratios in TB cases were due to the effect of the active disease process itself, TB cases were stratified into Active and Past History and compared with healthy controls, shown in figure 5.6 (b). There was a gradient of responses, with Active TB cases having lower values compared with Past History cases and controls. There was no significant difference in $\text{TNF}\alpha$ ratios between Past History cases and controls.

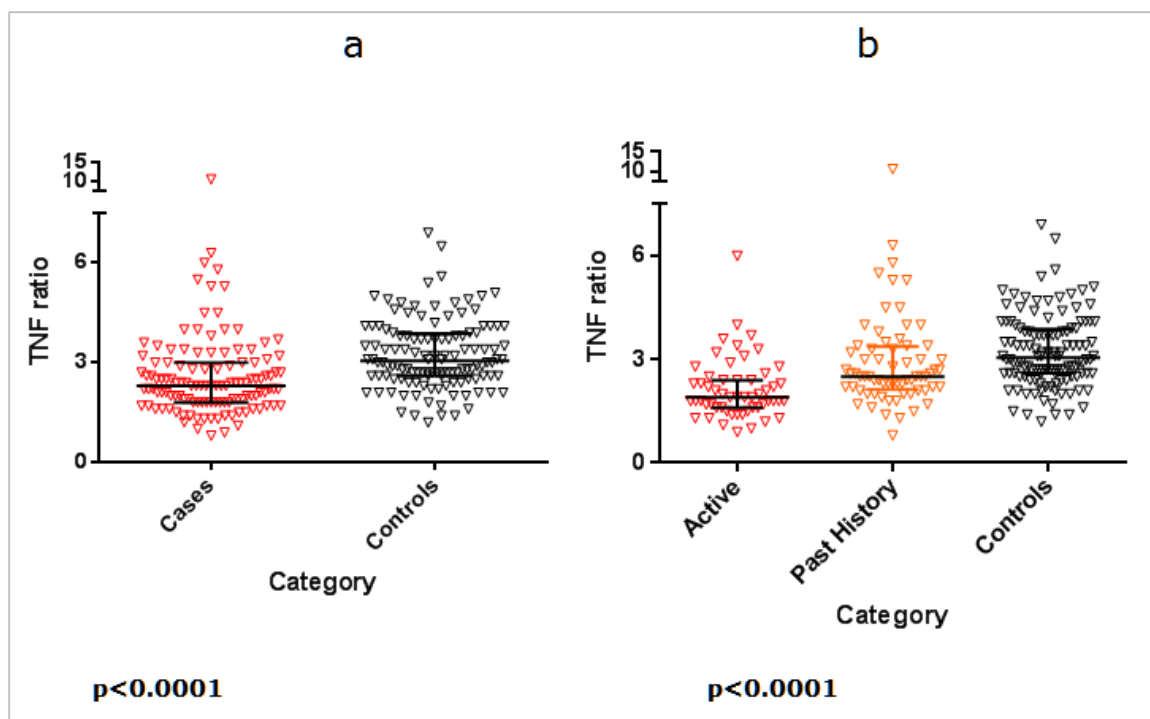


Figure 5.6: TNF α ratios (stimulation with LPS +IFN γ /LPS) indicative of response to IFN γ , in TB cases and healthy controls.

Notes:

a: Values shown for Cases: (n= 123) median 2.3 IQR (1.8-3); Controls: (n=127) median 3.1 IQR (2.6-3.9). p value is for Mann-Whitney U test.

b: Values shown for Cases Active: (n=55) median 1.9 IQR (1.6-2.4); Cases Past History: (n=68) median 2.5 IQR (2.15-3.35); Controls: (n=127) median 3.1 IQR (2.6-3.9). p value is for Kruskal Wallis one-way analysis for variance of ranks.

5.4.3.3 IL12p70 responses

IL12p70 responses of all TB cases, Active and Past History cases combined, were significantly lower than those of the healthy controls ($p<0.0001$), shown in figure 5.7 (a). To evaluate if the lower IL12 responses in TB cases were due to the effect of the active disease process itself, TB cases were stratified into Active and Past History and compared with controls, shown in figure 5.7 (b). A significant difference between stratified cases and controls was observed

($p < 0.0001$), suggesting that patients with TB were phenotypically different from healthy controls in their ability to upregulate IL12.

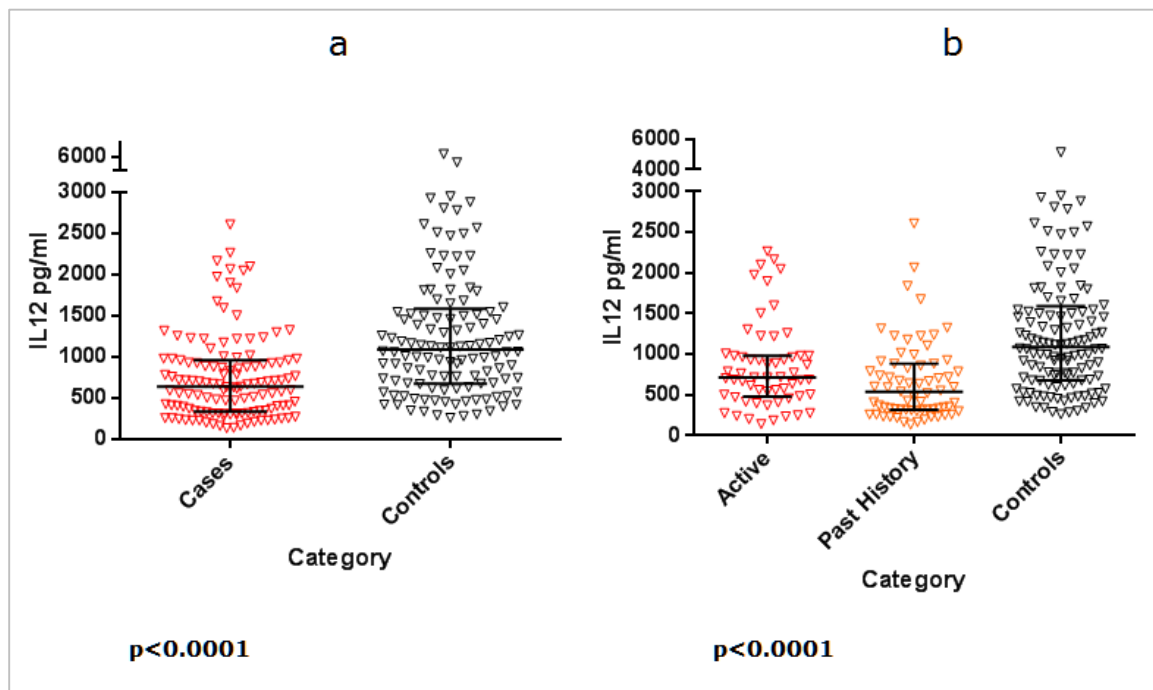


Figure 5.7: IL12p70 responses (pg/ml) to LPS+IFN γ indicative of ability to produce IL12, in TB cases and healthy controls.

Note:

a: Values shown for Cases: (n=127) median 641 IQR (388-962); Controls: (n=124) median 1092 IQR (680-1577). p value is for Mann-Whitney U test.

b: Values shown for Cases Active: (n=58) median 715 IQR (486-983); Cases Past History: (n=69) median 540 IQR (318-883); Controls: (n=124) median 1092 IQR (680-1577). p value is for Kruskal Wallis one-way analysis for variance of ranks.

Surprisingly, a significant difference was seen in IL12p70 production between Active and Past History TB cases ($p=0.023$), shown in figure 5.8 (c).

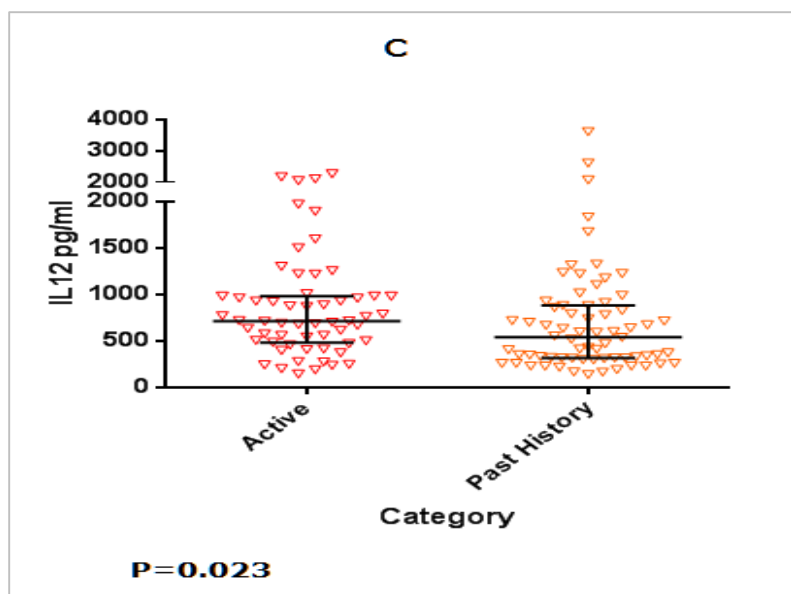


Figure 5.8: IL12p70 responses (pg/ml) to LPS+IFN γ , indicative of ability to produce IL12, in TB cases (active and past history).

Note:

c: Values shown for Cases Active: (n=58) median 715 IQR (486-983); Cases Past History: (n=69) median 540 IQR (318-883). p value is for Mann-Whitney U test.

5.4.3.4 IFN γ responses

For analysis of IFN γ responses to PHA, TB cases were compared with healthy controls with evidence of TB infection. Healthy controls were therefore stratified by TST response and only those with measurements of ≥ 10 mm used.

5.4.3.4.1 IFN γ responses to PHA in TB cases and healthy controls

There was no significant difference in IFN γ responses to PHA between all TB cases and healthy controls, but on stratifying cases into Active and Past History and comparing with controls, the Active cases had significantly higher values (p=0.003) shown in figure 5.9 (a and b).

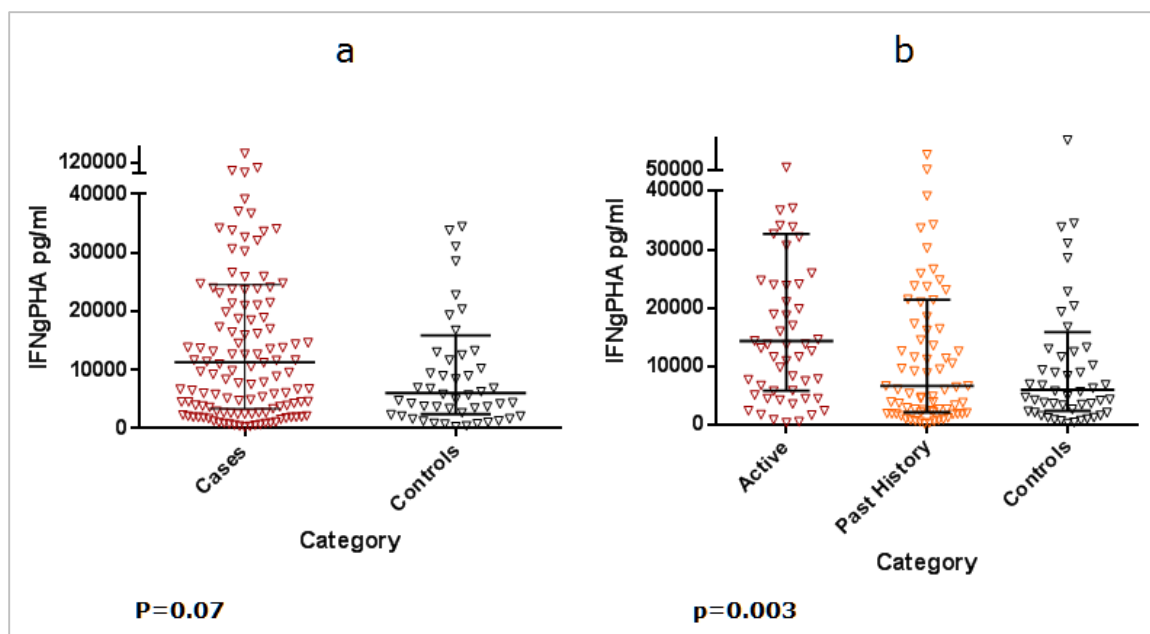


Figure 5.9: IFN γ responses (pg/ml) to PHA in TB cases and healthy controls.

Notes:

a: Values shown for Cases: (n=136) median 11320 IQR (3502-24414); Controls: (n=52) median 6066 IQR (2523-15052). p value is for Mann-Whitney U test.

b: Values shown for Cases Active: (n=59) median 14396 IQR (5903-32577); Cases Past History: (n=77) median 6719 IQR (2300-21387); Controls: (n=52) median 6066 IQR (2523-15052). p value is for Kruskal Wallis one-way analysis for variance of ranks.

When comparing Active and Past History TB cases, each with healthy controls, the Active cases were again seen to have higher IFN γ levels than the healthy controls (p=0.002), shown in figure 5.10 (c). No difference was seen with comparison of Past History cases and healthy controls, shown in figure 5.10 (d).

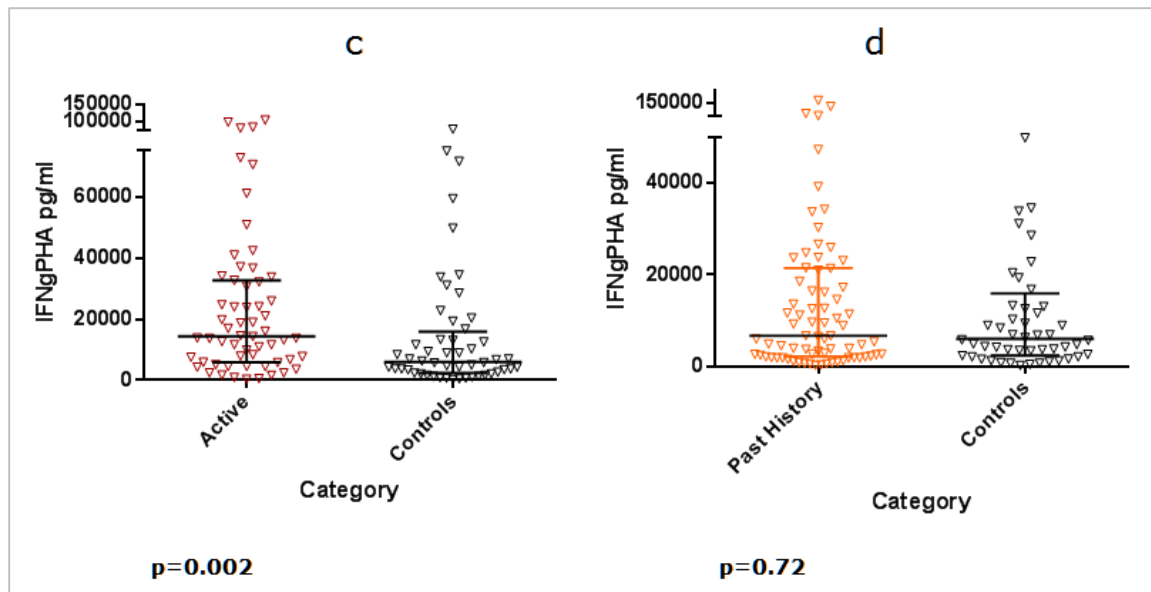


Figure 5.10: IFN γ responses (pg/ml) to PHA in TB cases (active and past history) and healthy controls.

Notes:

c: Values shown for Cases Active: (n=59) median 14396 IQR (5903-32677); Controls: (n=52) median 6066 IQR (2523-15052). p value is for Mann-Whitney U test.

d: Values shown for Cases Past History: (n=77) median 6719 IQR (2300-21387); Controls: (n=52) median 6066 IQR (2523-15052). p value is for Mann-Whitney U test.

To test the responsiveness to IFN γ production to IL12, the additive effect of IL12 with PHA was used as stimulus for IFN γ . This was expressed as a ratio of IFN γ in response to PHA+IL12/PHA, shown in figure 5.11 (e and f). No difference was seen when comparing cases with controls.

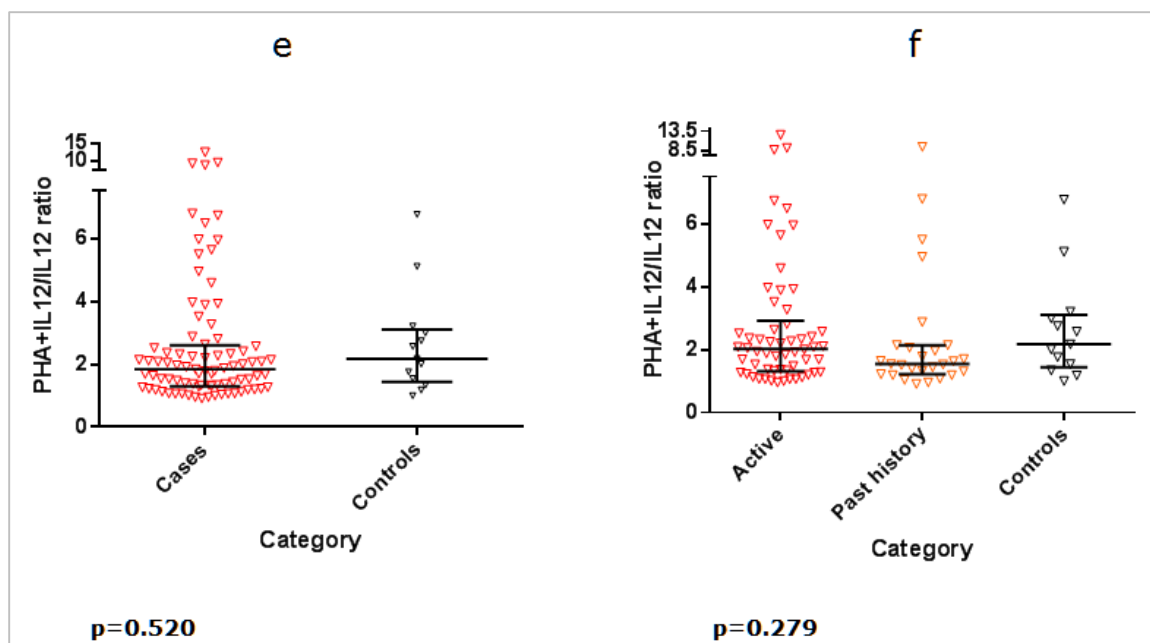


Figure 5.11: Ratios of IFN γ responses to PHA+IL12/PHA, indicative of ability to respond to IL12, in TB cases and healthy controls.

Notes:

e: Values shown for Cases: (n=85) median 1.84 IQR (1.31-2.57); Controls: (n=14) median 2.09 IQR (1.35-3.0). p value is for Mann-Whitney U test.

f: Values shown for Cases Active: (n=58) median 2.03 IQR (1.33-2.81); Cases Past History: (n=27) median 1.54 IQR (1.22-2.14); Controls: (n=14) median 2.09 IQR (1.35-3.0). p value is for Kruskal Wallis one-way analysis for variance of ranks.

5.4.3.4.2 IFN γ responses to PPD in TB cases and healthy controls

Predictably, all TST positive healthy controls produced IFN γ in response to PPD. On comparison with TB cases, the TST positive controls had significantly higher values than the cases (p=0.002) shown in figure 5.12 (a). When TB cases were stratified into Active and Past History cases, similar IFN γ responses to PPD were observed, with controls having higher levels (p=0.0009), shown in figure 5.12 (b).

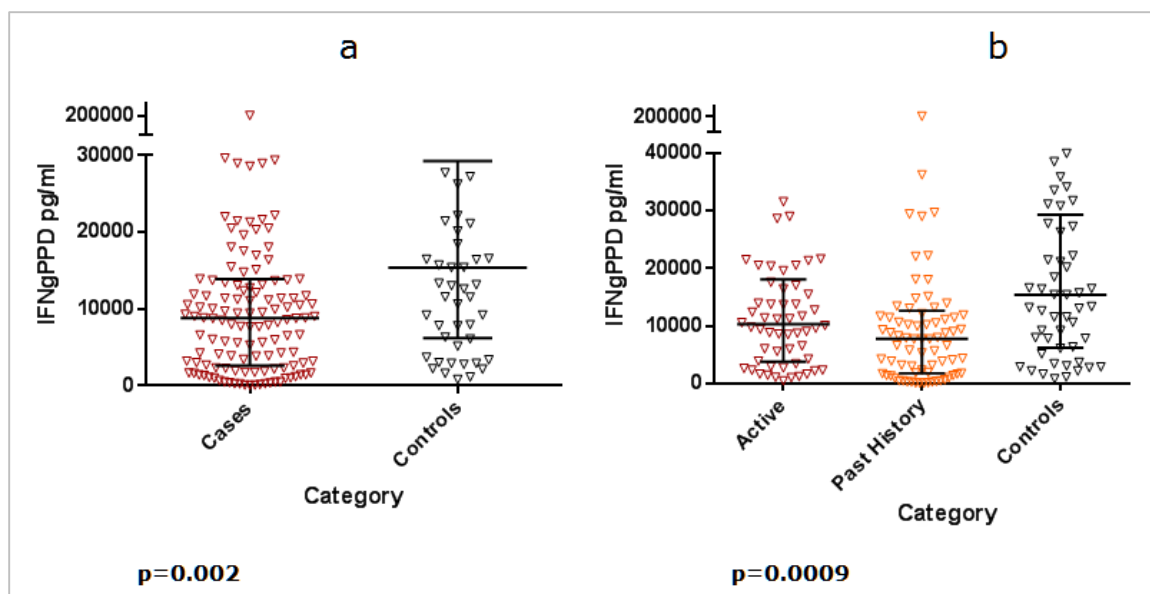


Figure 5.12: IFN γ responses (pg/ml) to PPD in TB cases and healthy controls.

Note:

a: Values shown for Cases: (n=135) median 8856 IQR (2624-13923); Controls: (n=52) median 15402 IQR (6360-27747). p value is for Mann-Whitney U test.

b: Values shown for Cases Active: (n=58) median 10313 IQR (3916-17547); Cases Past History: (n=77) median 7744 IQR (1797-12160); Controls (n=52) median 15402 IQR (6360-27747). p value is for Kruskal Wallis one-way analysis for variance of ranks.

Stratifying TB cases into Active and Past history and comparing with healthy controls demonstrated significantly higher IFN γ responses in controls compared with Past History cases (p=0.0004). While there was a trend for lower values in Active cases, this did not reach significance (p=0.066), shown in figure 5.13 (c and d).

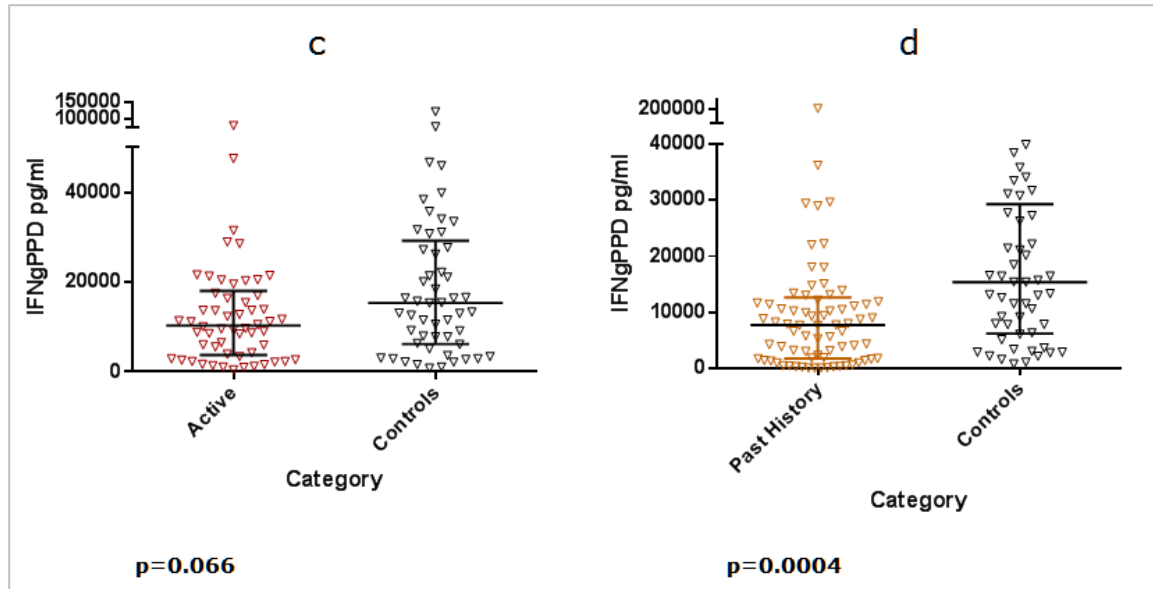


Figure 5.13: IFN γ responses (pg/ml) to PPD in TB cases (active and past history) and healthy controls.

Notes:

c: Values shown for Cases Active: (n=58) median 10313 IQR (3916-17547); Controls (n=52) median 15402 IQR (6360-27747). p value is for Mann-Whitney U test.

d: Values shown for Cases Active: (n=58) median 10313 IQR (3916-17547); Controls (n=52) median 15402 IQR (6360-27747). p value is for Mann-Whitney U test.

5.4.3.5 Role of SNPs on cytokine responses

To determine if the identified SNPs in the IL12p40 promoter had a functional effect on cytokine production, comparison was made of cytokine responses in TB cases with SNP genotypes and wildtype healthy controls and of cytokine responses in healthy controls with SNP genotypes and wildtype TB cases.

5.4.3.5.1 Effect of SNPs on IL12p70 responses

Comparison of IL12p70 responses in wildtype healthy controls and TB cases with SNP genotypes showed no significant difference between genotypes, as shown in figure 5.14 (a and b).

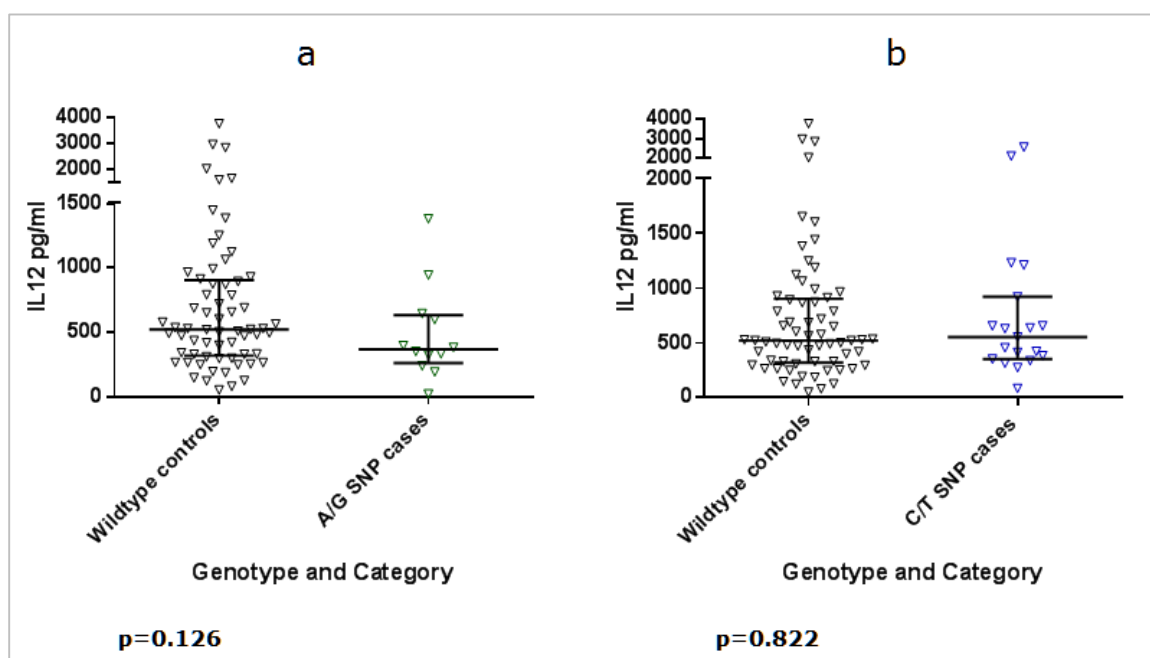


Figure 5.14: IL12p70 responses (pg/ml) wildtype healthy controls and TB cases with SNP genotype.

Note:

a: Values shown for Wildtype Controls: (n=65) median 522 IQR (329-893); Genotype -1523 A/G Cases: (n=12) median 367 IQR (281-621). p value is for Mann-Whitney U test.

b: Values shown for Wildtype Controls: (n=65) median 522 IQR (329-893); Genotype -1564 C/T Cases: (n=20) median 507 IQR (346-790). p value is for Mann-Whitney U test.

Comparisons of IL12p70 responses in wildtype TB cases and healthy controls with SNP genotypes showed no significant difference between genotypes, as shown in figure 5.15 (c and d). The number of controls

with SNP genotype that had IL12 levels measured was less than that for other cytokines, resulting in lower numbers for analysis.

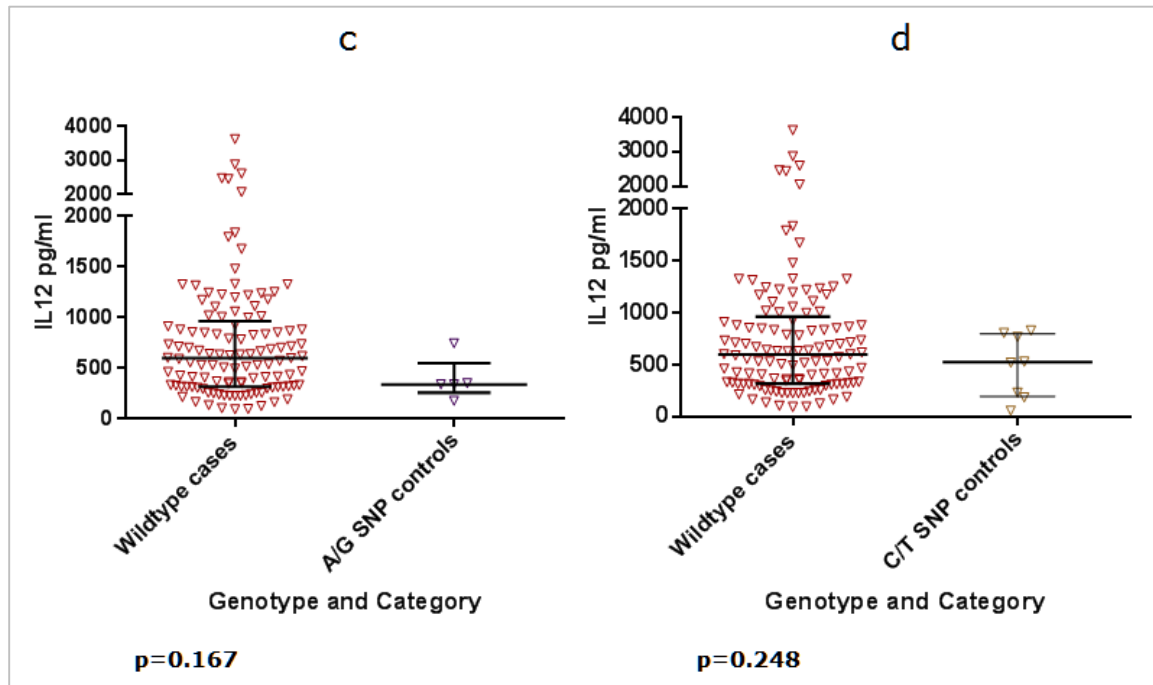


Figure 5.15: IL12p70 responses (pg/ml) in wildtype TB cases and healthy controls with SNP genotype.

Notes:

c: Values shown for Wildtype Cases: (n=117) median 601 IQR (324-928); Genotype -1523 A/G Controls: (n=5) median 342 IQR (342-355). p value is for Mann-Whitney U test.

d: Values shown for Wildtype Cases: (n=117) median 601 IQR (324-928); Genotype -1564 C/T Controls: (n=8) median 529 IQR (211-790). p value is for Mann-Whitney U test.

5.4.3.5.2 Effect of SNP on IFN γ responses to PHA

Comparison IFN γ responses to PHA in wildtype healthy controls and TB cases with SNP genotypes showed no significant differences between genotypes, as shown in figures 5.16 (a and b).

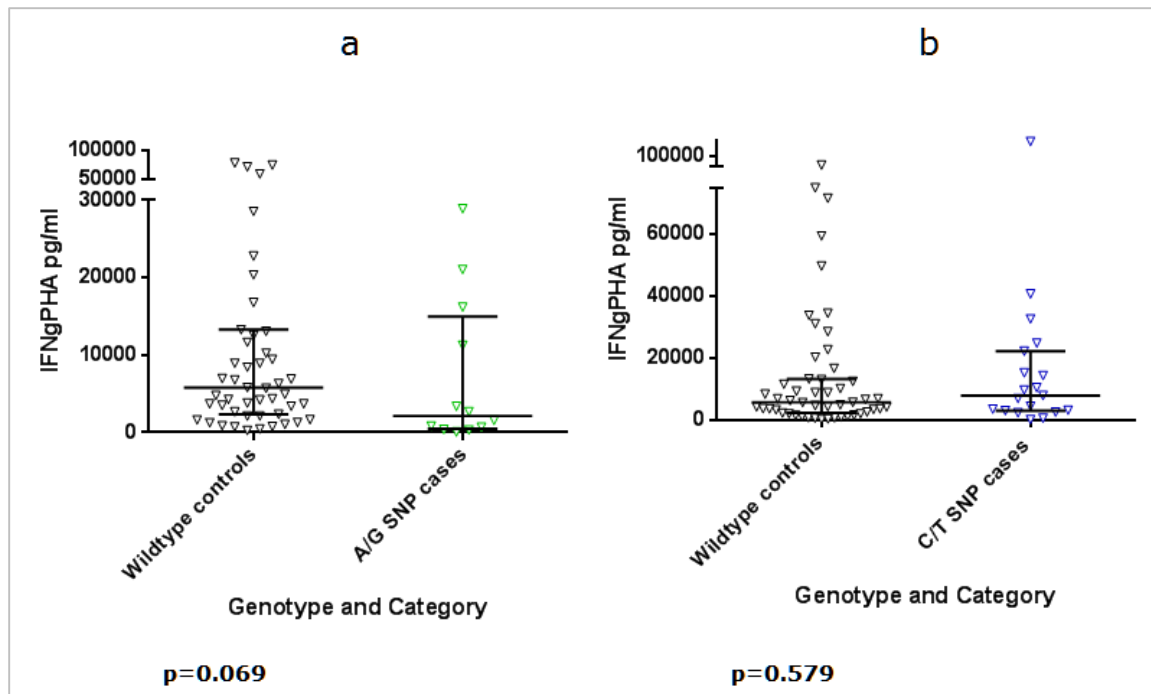


Figure 5.16: IFN γ responses (pg/ml) to PHA in wildtype healthy controls and TB cases with SNP genotype.

Notes:

a: Values shown for Wildtype Controls: (n=52) median 5810 IQR (2362-13289); Genotype -1523 A/G Cases: (n=12) median 2156 IQR (556-13746). p value is for Mann-Whitney U test.

b: Values shown for Wildtype Controls: (n=52) median 5810 IQR (2362-13289); Genotype -1564 C/T Cases: (n=20) median 8022 IQR (3138-22246). p value is for Mann-Whitney U test.

Comparison of IFN γ responses to PHA in wildtype TB cases and healthy controls with SNP genotypes and showed no significant difference between genotypes for the -1523 A/G SNP, shown in figure 5.17 (c). However, wildtype TB cases were shown to have higher IFN γ levels than the healthy controls with -1564 C/T genotype (p=0.01), shown in figure 5.17 (d).

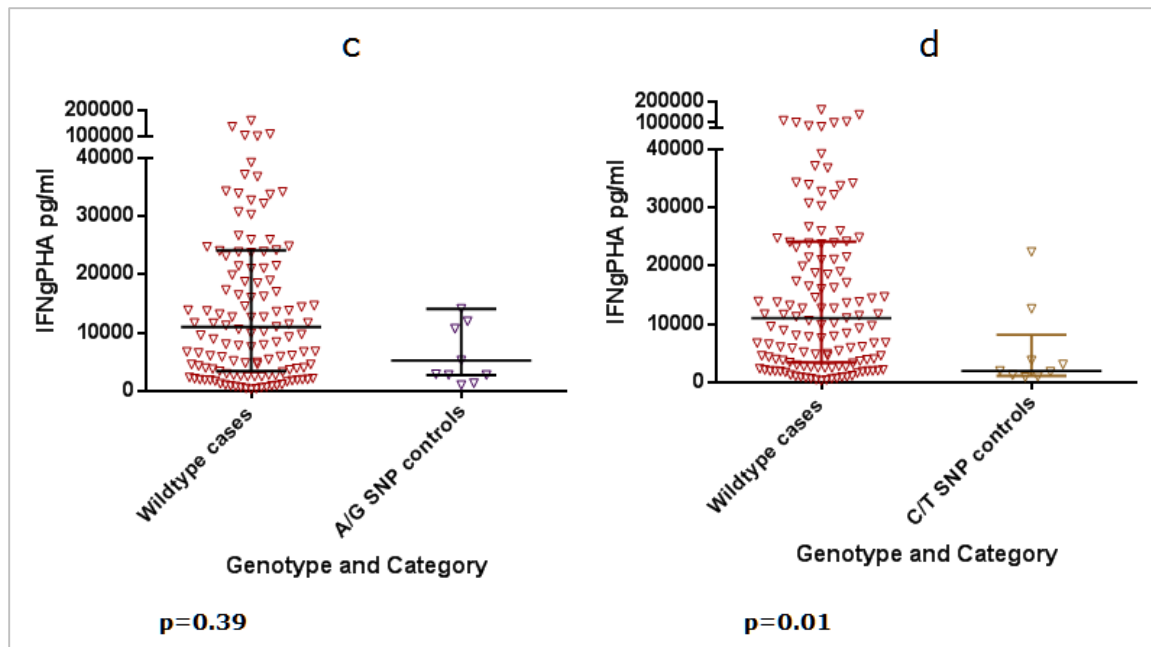


Figure 5.17: IFN γ responses (pg/ml) to PHA in wildtype TB cases and healthy controls with SNP genotype.

Notes:

c: Values shown for Wildtype Cases (n=136) median 11320 IQR (3502-2441); Genotype -1523 A/G Controls (n=10) median 5226 IQR (2760-14082). p value is for Mann-Whitney U test.

d: Values shown for Wildtype Cases: (n=136) median 11320 IQR (3502-24414); Genotype -1564 C/T Controls: (n=9) median 2488 IQR (1503-8145). p value is for Mann-Whitney U test.

5.4.3.5.3 Effect of SNPs on IFN γ responses to PPD

Comparison of IFN γ responses to PPD in wildtype healthy controls and TB cases with SNP genotypes showed no difference for the -1523 A/G SNP, but TB cases with -1564 C/T genotype were shown to have lower IFN γ levels than the wildtype healthy controls (p=0.002), shown in figure 5.18 (a and b).

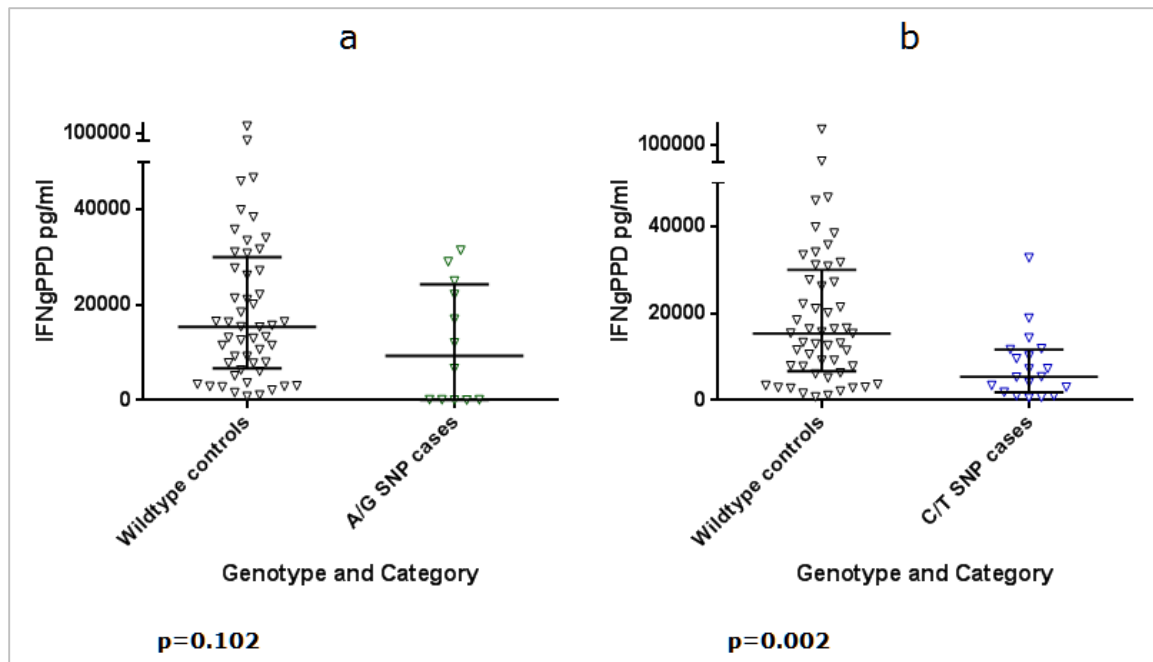


Figure 5.18: IFN γ responses (pg/ml) to PPD in wildtype healthy controls and TB cases with SNP genotype.

Notes:

a: Values shown for Wildtype Controls: (n=52) median 15417 IQR (7090-29279); Genotype -1523 A/G Cases: (n=12) median 9360 IQR (112-23619). p value is for Mann-Whitney U test.

b: Values shown for Wildtype Controls: (n=52) median 15417 IQR (7090-29279); Genotype -1564 C/T Cases: (n=20) median 5429 IQR (1876-11691). p value is for Mann-Whitney U test.

Comparison of IFN γ responses to PPD in wildtype TB cases and healthy controls with SNP genotypes showed no differences for the -1523 A/G SNP, but healthy controls with -1564 C/T genotype were seen to have lower levels than wildtype TB cases (p=0.0005), shown in figure 5.19 (c and d).

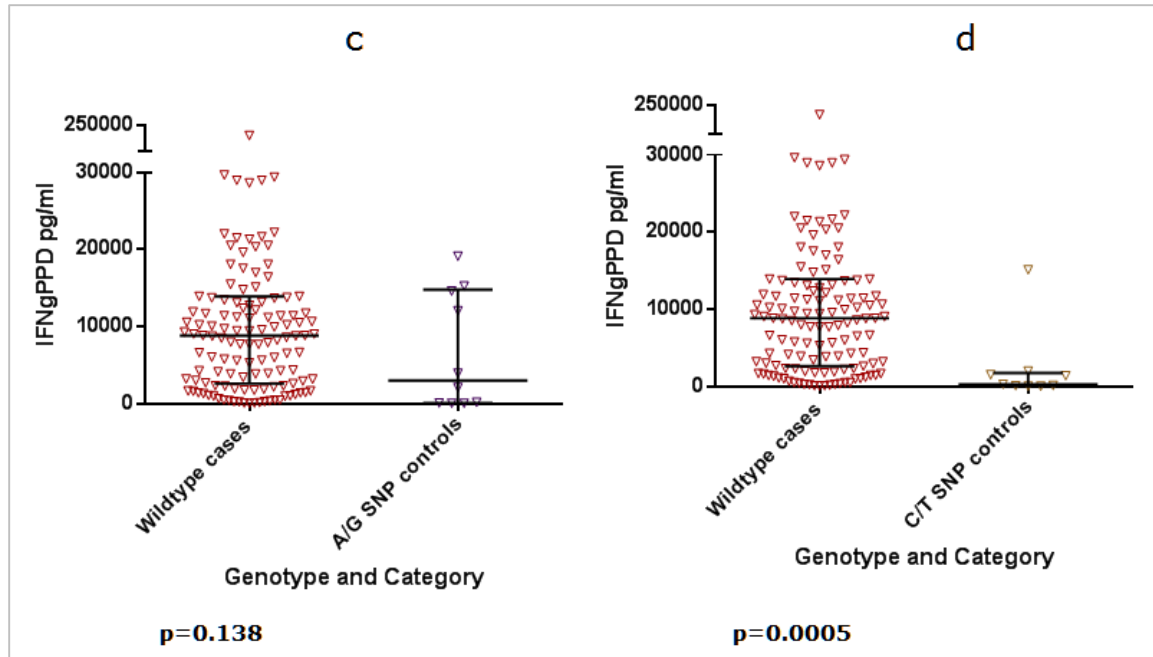


Figure 5.19: IFN γ responses (pg/ml) to PPD in wildtype TB cases and healthy controls with SNP genotype.

Notes:

c: Values shown for Wildtype Cases: (n=135) median 8856 IQR (2624-13923); Genotype -1523 A/G Controls (n=10) median 3962 IQR (92-14620). p value is for Mann-Whitney U test.

d: Values shown for Wildtype Cases: (n=135) median 8856 IQR (2624-13923); Genotype -1564 C/T Controls: (n=9) median 300 IQR (94-1526). p value is for Mann-Whitney U test.

5.5 Measurement of IL12R β 1 expression by Flow Cytometry.

IL12R β 1 cell surface expression was measured by flow cytometry, using PBMCs isolated from heparinized blood, stimulated with PHA and labelled with a fluorescent IL12R β 1 antibody (Ozenci *et al.* 2001, Zaki *et al.* 2001) as detailed in methods, section 6.4.

5.5.1 IL12R β 1 expression

Cell surface expression of the IL12R β 1 cytokine receptor is shown in figure 5.20.

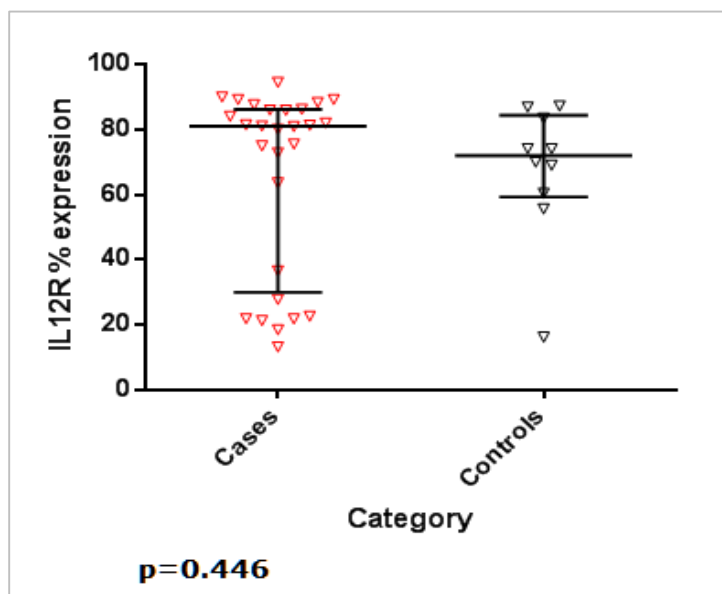


Figure 5.20: IL12R β 1 expression (percentage) in TB cases and healthy adult controls.

Note:

Values shown for Cases: (n=31) median 81 IQR (32-86); Adult Controls: (n=10) median 72 IQR (60-84). Value is for Mann-Whitney U test.

5.6 Discussion

Diagnosis of Mendelian susceptibility to mycobacterial infection remains challenging. At the time of completing this work, the technology for rapid sequencing of genes and whole exome sequencing was not available in Cape Town nor developed to the extent that it is today (2013).

To help tease out the site of a genetic mutation in patients suspected to have this disorder, WB cytokine stimulation assays and

measurement of receptor expression can be used to probe the site of a suspected defect. Thereafter, screening for mutations in a suspected gene or genes can be completed making it a rapid and relatively inexpensive screening tool.

In this chapter I have described the screening process for defects in the $\text{IFN}\gamma$ pathway, by measurement of $\text{TNF}\alpha$, IL12p70 and $\text{IFN}\gamma$ after stimulation in WB assays and measurement of IL12R β 1 by flow cytometry. Cytokines do not function in isolation, but operate within a complex network. IL12 plays a pivotal role in cell mediated immunity, enhancing the cytotoxic activity of NK and T cells and stimulating $\text{IFN}\gamma$ production. The effects and responses of $\text{TNF}\alpha$, IL12 and $\text{IFN}\gamma$ all interact within this cytokine network.

Because of small numbers of children recruited from the Cape Coloured population, stratification by ethnicity was not carried out.

Stratification by age and gender were also not done, as the aim of this work was to identify an immune phenotype resulting in susceptibility to TB, not affected by age or gender.

On the immune testing of this paediatric cohort screened, various cytokine profiles were seen. Overall responses were heterogeneous for both TB cases and healthy controls.

The lower $\text{TNF}\alpha$ ratios seen in all TB cases compared to higher ratios seen healthy controls ($p < 0.0001$), is probably due to the mycobacterial infection. When the TB cases were stratified into Active and Past History cases and compared to the healthy controls, this lower response in cases was still evident. The reason for this was due to the fact that TB cases (both Active and Past History) produced higher levels of $\text{TNF}\alpha$ to LPS alone, compared to healthy controls (data

not shown). The reasons for this are unclear, but may relate to the fact that TB disease has switched on a more profound inflammatory response in cases compared to healthy controls and that this effect persists even after completion of TB treatment.

The lower IL12 levels seen in all TB cases as a combined group and when stratified into Active and Past History cases, compared to healthy controls ($p < 0.0001$), is also probably due to the clinical disease, evident even 12 months after diagnosis. In retrospect, it would have been best to measure IL12p40, but at the time, antibodies to the heterodimer IL12p70 were readily available, so this was measured.

Analysis of IFN γ production in response to PHA, showed no significant difference between TB cases as a combined group and controls. This was not unexpected and is likely due to the fact that PHA is a T cell mitogen, used to assess non specific responsiveness to IFN γ . However, on stratifying into groups, Active TB cases were shown to have higher levels of IFN γ ($p = 0.003$). Comparisons of IFN γ responses to PHA in Active and Past History TB cases, each with healthy controls, showed this difference still to be evident ($p = 0.002$). This is likely to be due to an enhanced immune response in the Active TB cases.

PPD is used to specifically look at IFN γ responses to mycobacterial antigens. Analysis of IFN γ production in response to PPD, showed a significant difference between TB cases as a combined group ($p = 0.002$), or stratified into Active and Past History cases ($p = 0.0009$), compared to healthy controls. The healthy controls had higher responses compared to the cases. This was likely due to the fact that all IFN γ producing cells are at the site of disease in the lung and not in peripheral circulation (Sutherland *et al.* 2012). Another reason for this result could be that patients with TB are phenotypically different to

healthy controls and less able to upregulate $\text{IFN}\gamma$ and are therefore more susceptible to TB.

Analysis of $\text{IL12R}\beta 1$ expression showed no absence of receptor expression. However, there was a clear bimodal distribution of receptor expression with a cluster of seven patients seen to have very low expression following stimulation with PHA. In contrast, among the 10 healthy controls screened, only one individual was found to have low expression. There are a number of reasons that may have accounted for this finding. First, this may be a normal variant with no functional effect. Very few controls were screened, all of whom were healthy adult lab volunteers with no past history of mycobacterial disease. With larger numbers, more individuals with low expression may have been found. Second, low expression may be associated with a functional inability to effectively upregulate $\text{IFN}\gamma$. One way to evaluate absent or abnormally responsive $\text{IL12R}\beta 1$ expression is to measure $\text{IFN}\gamma$ production to PHA in the presence and absence of exogenous IL12 (section 6.2.1.2.1). Unfortunately assays for both $\text{IL12R}\beta 1$ expression and IL12 ratio were set up at different time points and as a result there were very few patients with both sets of data for comparison. Correlation of larger numbers would be an opportunity for future work.

Comparison of $\text{IFN}\gamma$ responses to PHA, expressed as a ratio of $\text{PHA}+\text{IL12}/\text{PHA}$, showed no difference between cases and healthy controls.

Analysis of the role of the SNPs on cytokine responses showed varied results.

No differences between wildtype and SNP genotypes were seen on analysis of IL12 levels.

On analysis of the functional effects of the SNPs with respect to IFN γ production, results were varied. Analysis of IFN γ production in response to PHA showed the wildtype TB cases to have higher IFN γ levels than the healthy controls with -1564 C/T genotype ($p=0.01$).

Analysis of IFN γ production in response to PPD showed significant differences between genotypes. The TB cases with -1564 C/T genotype were seen to have lower IFN γ levels than the wildtype healthy controls ($p=0.002$) and the healthy controls with -1564 C/T genotype were also found to have lower levels than the wildtype TB cases ($p=0.0005$).

These results are to be interpreted with caution as this variation of results is likely to be due to the very small numbers of individuals with SNPs, resulting in a lack of power for accurate statistical analysis.

In conclusion, an immune phenotype correlating with the SNPs found in the IL12p40 gene promoter was not observed. The reasons for this may be due to a number of reasons: the small numbers of subjects identified with SNPs and associations may have arisen by chance.

Studies using larger numbers would be valuable.

CHAPTER 6: General Methods

The study group and ethics are discussed in chapter 3.

6.1 Clinical Methods

6.1.1 Mantoux testing

6.1.1.1 Method

Purified protein derivative of *M. tb* (PPD), batch RT-23, (Statens Serum Institute, Copenhagen, Denmark) was used. Two tuberculin units (0.1ml) were injected intradermally on the volar surface of the left forearm. PPD was injected using a tuberculin syringe (Beckton Dickenson, Oxford UK). This procedure was done by one experienced nurse.

6.1.1.2 Measurement of Mantoux reactions

Induration diameters were measured 48-72 hours later using the ballpoint pen technique to determine the borders of the indurated area. This was completed by one nurse who administered the tuberculin and the measurement was verified by her co-worker. The widest transverse diameter of the induration was recorded in mm. Where no palpable induration was detectable, the result was recorded as '0'. Mantoux responses of 15 mm or greater were categorized as positive, while those of less than 10 mm were categorized as negative. Any child with an induration diameter of 15 mm or greater was referred to the local TB clinic for a chest X-ray and assessment in accordance with national guidelines (The South African Tuberculosis Control Program Guidelines, 2000).

6.2 Cellular Methods

6.2.1 Measurement of immunological phenotype in cases and controls

Blood samples were handled under strict tissue culture conditions in a class II bio-safety cabinet to avoid contamination. For all whole blood assays (WBA), venous blood was collected into a sterile syringe containing preservative free sodium heparin (Sigma Cat. No. H3149) (10ul/ml blood = 10 U/ml blood) and transferred into a sterile tube (10ml Falcon Cat. No. 128B).

For the cytokine assays, bloods were diluted 1:10 in sterile plastic tubes (7ml Bijou Cat. No. DA275046024) in tissue culture medium (6.3ml tissue culture medium + 0.7ml blood) composed of:

RPMI 1640 with 1% L-glutamine (200mM) (Gibco Cat. No. 72400-021), penicillin (100IU/ml) and streptomycin (100mg/ml) (Pen-Strep)(Gibco Cat. No. 15140-114).

A blood sample from a pool of healthy adult volunteers was included in every upregulation experiment.

6.2.1.1 Production of TNF α and IL12p70 in whole blood

A WBA for TNF α upregulation using LPS of E. Coli and IFN γ together to enhance TNF α production, was used to screen for IFN γ responsiveness. This was an adaptation of the method developed and used by Levin and colleagues (Levin et al. 1995) to investigate children for defects in the IFN γ R1 gene. This was reoptimized to enable harvesting of supernatants following an overnight incubation and the reproducibility verified on a group of healthy laboratory controls. The concentration of assay reagents and time point for aspiration of supernatants was

chosen following experiments adapting a WBA tube method into a 96 well plate assay and assessing the time course of peak $\text{TNF}\alpha$ production following stimulation with LPS and $\text{IFN}\gamma$.

6.2.1.1.1 Reagents

For the $\text{TNF}\alpha$ assay, *E. coli* 0111:B4 LPS (Sigma Cat. No. L2630) was supplied as 10mg lyophilised powder and reconstituted in 1ml RPMI to give a final concentration of 10mg/ml. This was further diluted 1:1000 in RPMI to give a stock concentration of 10 $\mu\text{g}/\text{ml}$ which was stored in 100 μl aliquots at -80°C . A working solution of LPS was freshly prepared from frozen stock on the day of WBA by diluting stock LPS 1:2000 in RPMI to give a final concentration of 5ng/ml. Recombinant Human $\text{IFN}\gamma$ (rHu $\text{IFN}\gamma$) (Immukin, Boehringer Ingelheim Cat. No. PL00015/0154) was prepared from stock at 100 $\mu\text{g}/0.5\text{ml}$ diluted 1:100 in PBS to give a final concentration of 2000ng/ml). The working solution was stored at 4°C for a maximum of one month.

A similar WBA for IL12 upregulation using LPS of *E. coli* and $\text{IFN}\gamma$ was used to screen for production of IL12p70. For the IL12p70 assay a working solution of LPS was freshly prepared from frozen stock on the day of WBA by diluting stock LPS 1:100 in RPMI to give a final concentration of 100ng/ml. Stock $\text{IFN}\gamma$ (100 $\mu\text{g}/0.5\text{ml}$) was diluted 1:10 in PBS to give a final concentration of 20 $\mu\text{g}/\text{ml}$. The working solution was stored at 4°C for a maximum of one month.

6.2.1.1.2 Method for $\text{TNF}\alpha$ and IL12p70 upregulation $\text{TNF}\alpha$ upregulation

Diluted blood (180 μl) was placed into 12 wells of a 96 well, round-bottomed tissue culture plates (Greiner Cat. No. 163320). $\text{IFN}\gamma$ at a final concentration of 100ng/ml was added to the first four wells and

an equivalent volume of RPMI added to a further 6 wells. The plates were then incubated at 37°C in 5% CO₂ for two hours. LPS at a final concentration of 0.5ng/ml was added to four wells 1-4 containing IFN γ or RPMI and the wells mixed 5 times with a multi-channel pipette. Control wells contained RPMI alone. Incubation was continued for a further 18 hours at 37°C in 5% CO₂. Supernatants from quadruplicate wells were harvested, pooled and stored at -80°C in two aliquots until analysis by ELISA. A summary of this procedure is shown in table 6.1.

TNFα plate plan	Wells 1-4	Wells 5-8	Wells 9-12
	Unstimulated	LPS	IFN γ + LPS
RPMI	30ul	10ul	-
IFN γ 1:100	-	-	10ul
Blood 1:10	180ul	180ul	180ul
LPS 1:2000	-	20ul	20ul
NB: TNF α plate was incubated for 2 hours before the addition of LPS			

Table 6.1: Summary of loading volumes for TNF α upregulation in microtitre plate IL12p70 upregulation

The TNF α method was adapted for measuring IL12 up-regulation using IFN γ and LPS at final concentrations of 1 μ g/ml and 10ng/ml respectively. Control wells contained RPMI alone. Supernatants from quadruplicate wells were harvested, pooled and stored at -80°C as two duplicate aliquots until analysis by ELISA. The final concentration of assay reagents and the time course of peak IL12 production were chosen following optimisation experiments. A summary of this procedure is shown in table 6.2.

IL12p70 plate plan	Wells 1-4	Wells 5-8	Wells 9-12
RPMI	-		
IFN γ 1:10	10ul		
Blood 1:10	180ul		
LPS 1:100	20ul		
NB: IL12 plate was incubated for 2 hours before the addition of LPS			

Table 6.2: Summary of loading volumes for IL12 upregulation in microtitre plate

6.2.1.2 IFN γ production in whole blood

6.2.1.2.1 Reagents

Preparations of 100,000 units of PPD per ml (Evans Medical Limited, Leatherhead, UK) were diluted in RPMI to a final concentration of 10 μ g/ml ready for use. Aliquots were stored at -20°C and defrosted prior to use. PHA (Sigma Cat. No L-1668) was diluted in RPMI to a final concentration of 5 μ g/ml and used for WBA stimulation of IFN γ production. The solution was stored in aliquots at -20°C and defrosted immediately before use.

6.2.1.2.2 Method for IFN γ upregulation

Twenty microlitres of solution containing either PHA at 5 μ g/ml, PPD 10 μ g/ml or phosphate buffered saline (PBS) as unstimulated control were pipetted into 96 well, round-bottom tissue culture wells (Greiner Cat. No 163320). Four wells were set up for each stimulant with each blood sample. An additional 4 wells containing both PHA at 5 μ g/mL and recombinant human (rHu) IL12p70 (BD Pharmingen, Becton-Dickinson, Oxford, UK) at a final concentration of 50pg/ml were also included, as a screen for IL12 responsiveness. Diluted blood (180 μ l) was pipetted into each well. The plates were incubated at 37°C in a

5% CO₂ incubator. On day three, supernatants were harvested from the wells containing PHA/blood and the unstimulated control. On day six, supernatants were harvested from the wells containing PPD/blood and the unstimulated controls. Quadruplicate wells were harvested, pooled and stored as two duplicate aliquots at -80°C until analysis by ELISA.

Separate plates were used for PHA and PPD stimulation, due to their different incubation periods.

Optimal time for upregulation of IFN γ with PHA and PHA/IL12 was 3 days

Optimal time for upregulation of IFN γ with PPD was 6 days

Summaries of these procedures are shown in tables 6.3 and 6.4.

IFNγ PHA Plate Plan	Wells 1-4	Wells 5-8	Wells 9-12
	Unstimulated Control	PHA	PHA/IL12
RPMI	20ul	-	-
PHA 1:100	-	20ul	20ul
IL12 1:12500	-	-	10ul
Blood 1:10	180ul	180ul	180ul

Table 6.3: Summary of loading volumes for 3 day IFN γ upregulation with PHA in microtitre plate

IFN γ PPD Plate Plan	Wells 1-4	Wells 5-8
	Unstimulated Control	PPD
RPMI	20ul	-
PPD 1:20	-	20ul
Blood 1:10	180ul	180ul

Table 6.4: Summary of loading volumes for 6 day IFN γ upregulation with PPD in microtitre plate

Note: Rows A and H of the 96-well plate were not used to avoid excess evaporation during the long incubation periods.

Summary of loading volumes for 6 day IFN γ upregulation with PPD in microtitre plate

6.2.1.3 Production of pooled TNF α and IFN γ internal biological controls for ELISA

Using the TNF α and IFN γ upregulation assays described above, two batches of supernatant were collected for use as an internal quality control (QC) for measurement of inter-assay variation. In summary, 20ml of heparinized blood was collected from a single donor, diluted 1 in 10 with RPMI and 180 μ l aliquots pipetted into 96 well tissue plates. The blood was stimulated with either 5ng/ml of LPS plus 20ng of IFN γ or 5 μ g of PHA. TNF α and IFN γ supernatants were harvested and pooled after incubation at 37°C in 5% CO₂ for 18 hours and 3 days respectively. These were stored at -80°C in aliquots for use as ELISA controls.

6.3 Cytokine Measurement using ELISA technique

Concentrations of TNF α , IL12p70 and IFN γ in supernatants, collected at specific time points during WBA experiments, were measured using

sandwich ELISA antibody pairs according to manufacturer's recommendations.

6.3.1 Reagents

Coating buffer

ELISA plate coating buffer consisted of bicarbonate buffer (0.1 M Na_2HCO_3 pH 8.4) for $\text{TNF}\alpha$ and $\text{IFN}\gamma$ and sodium phosphate buffer (0.1M Na_2HPO_4 pH9.0) for IL12p70 assays.

Cytokine standards, purified mouse anti-human monoclonal capture and biotinylated detection antibodies were obtained from BD Pharmingen, details and catalogue numbers are listed in table 6.5.

Cytokine	Cytokine Standard	Capture Antibody	Detection Antibody
$\text{TNF}\alpha$	19761T	18631D	18642D
IL12p70	19721V	23271D	55466O
$\text{IFN}\gamma$	19751N	18891D	18902D

Table 6.5: Details of cytokine standards, capture and detection antibodies

PBS

This was prepared from 11.6g Na_2HPO_4 , 2.0g KH_2PO_4 , 2.0g KCl, 80.0g NaCl made up to 10L in Milli-Q H_2O (Invitrogen Cat. No. 10270), filtered with a 0.22 mm filter (Separations Inc. USA).

Blocker

10% Fetal Calf Serum (FCS) (Invitrogen Cat. No. 10270) was filtered with a 0.22 mm filter (Separations Inc. USA) prior to mixing with PBS.

Washing solution - PBS with 0.5% Tween 20

5.0ml Tween 20 was added to 10L of prepared PBS.

Diluent for samples, detection antibody and avidin peroxidase

10% FCS in PBS without Tween.

Avidin Peroxidase

Avidin peroxidase (Sigma A-3151) was stored at 1 mg/ml in sterile PBS at -80°C and diluted 1:1000 in 10% FCS/PBS for use. Aliquots were stored at -20°C and defrosted immediately prior to use.

Dilutions were made and final concentrations of 1.25µg/ml for TNF α , 3.3µg/ml for IL12 and 5µg/ml for IFN γ ELISA assays used.

Substrate

Substrate consisted of O-phenylenediamine dihydrochloride (OPD) (Sigma P-1526) dissolved in citric acid buffer (citric acid 5.19g/L, Na₂HPO₄.2H₂O 9g/L, pH 5.0 plus 10 ml of 30% H₂O₂)

4mg OPD was used per 10ml citric acid buffer.

Stop Solution

Stopping solution consisted of 2M H₂SO₄.

6.3.2 Method

Measurement of the TNF α , IL12p70 and IFN γ cytokines was a three day assay.

Day 1 – capture antibody

On day one, flat bottomed, 96-well high binding ELISA plates (Nunc 442404) were coated with 50µl per well of the capture antibody diluted in sodium bicarbonate buffer to a final concentration of 2µg/ml for TNF α and IFN γ and in sodium phosphate buffer at 5µg/ml for IL12p70. Plates were covered and kept at 4°C overnight.

Day 2 – blocking and loading with standards, samples and controls

Plates were washed twice in PBS with 0.5% Tween 20. Plates were blocked for 1-2 hours using 200 µl/well of PBS/10% FCS without Tween. After blocking, they were washed twice more with PBS/0.5% Tween 20 before application of samples and standards.

6.3.2.1 Preparation of cytokine standards for ELISA assay

Recombinant human cytokines (rHu) were used as reference standards.

Prior to use, the TNF α standard was diluted to 1µg/ml, IFN γ to 25µg/ml, and IL12p70 to 12.5µg/ml in 10mg/ml Bovine Serum Albumin in PBS, aliquoted and stored at -80°C.

For each experiment, one vial of each reconstituted, rHu cytokine was defrosted and diluted further for use.

One hundred microlitres of standard were applied in duplicate and serially diluted between 5000pg/ml and 19.5pg/ml for TNF α , 2000pg/ml and 7.8pg/ml for IL12 and 5000pg/ml and 19.5pg/ml for IFN γ across the ELISA plate. Where possible the same batch of standard was used for all experiments for the duration of the study.

6.3.2.2 Preparation of harvested supernatants

Frozen supernatants, generated during WBA experiments, were thawed at room temperature (RT), mixed by vortexing and 100 μ l serially diluted in 100 μ l volumes of PBS/10% FCS in duplicate.

Sample dilutions of harvested supernatants used are shown in table 6.6.

	TNF α LPS stimulation	TNF α LPS+IFN γ stimulation	IL12p70 LPS+IFN γ stimulation	IFN γ PHA stimulation	IFN γ PPD stimulation
Sample dilution	1 in 2 1 in 4	1 in 4 1 in 8	1 in 2	1 in 16 1 in 32 1 in 64	1 in 8 1 in 16 1 in 32

Table 6.6: Sample dilutions of supernatants used for cytokine measurement by ELISA

6.3.2.3 ELISA controls

6.3.2.3.1 WHO reference control

In order to control for any plate to plate as well as inter-assay variability WHO cytokine standards were used for each cytokine assayed. Recombinant human TNF α and IL12p70 standards were obtained from the National Institute for Biological Standards and Control (NIBSC) Potters Bar, Herts, UK (rHu TNF α : 87/650; rHu IL12p70: 95/544) and rHu IFN γ standard was obtained from the National Institute of Allergy and Infectious Diseases (NIAID) Reference Reagent Repository, Atlanta, USA (rHu IFN γ : Gxy01-902-535).

Prior to use, the $\text{TNF}\alpha$ reference control was diluted to $1\mu\text{g/ml}$, $\text{IFN}\gamma$ to $1\mu\text{g/ml}$, and IL12p70 to $0.5\mu\text{g/ml}$ in sterile water, aliquoted and stored at -80°C .

A single dilution of the WHO reference standard was applied to two wells on each plate per ELISA assay, to enable intra-plate variation to be quantified. The WHO standards were serially diluted on the plate to cover the following dilutions:

$\text{TNF}\alpha$ and $\text{IFN}\gamma$: 2000pg/ml to 62.5pg/ml . For IL12p70: 2000 pg/ml to 15pg/ml .

One concentration was applied to each plate per batch.

6.3.2.3.2 Internal biological controls

An in-house, pooled cytokine control supernatant, as described in Section 7.4.1.3 was also applied to two wells per ELISA batch for quantitation of inter-assay variation. These were used to ensure that results were comparable over an extended period of time.

ELISA plates were then sealed and incubated over night at 4°C .

Day 3 – completion of ELISA assay.

After 4 washes with PBS/0.5% Tween20, biotin labeled detection antibodies were diluted in 10% FCS in PBS with 0.5% Tween 20 to a final concentration of $1\mu\text{g/ml}$ for $\text{TNF}\alpha$ and $\text{IFN}\gamma$ and at $2.5\mu\text{g/ml}$ for IL12p70, and $100\mu\text{l}$ applied/well. After 45 minutes incubation at RT, the plates were washed 6 times and avidin peroxidase, at concentrations of $1.25\mu\text{g/ml}$ for $\text{TNF}\alpha$, $3.3\mu\text{g/ml}$ for IL12 and $5\mu\text{g/ml}$ for $\text{IFN}\gamma$, applied in a volume of $100\mu\text{l}$ to all wells and the plates incubated at RT for 30 minutes. After 8 further washes, ELISAs were

developed using 4mg of OPD per 10ml of citrate buffer with 10µl of 30% hydrogen peroxide, (pH5) as substrate in volumes of 100µl/well. Plates were incubated at RT for 20 minutes for IFN γ , 15 minutes for TNF and 40 minutes for IL12p70. The reaction was stopped using 100µl/well of H₂SO₄. Colour development was then read in a Versamax ELISA plate reader (Molecular Devices, Menlo Park, UK) and absorbance measured at OD 492 nm with a reference optical density of 620nm.

The ELISA method is summarized below in figure 6.1.

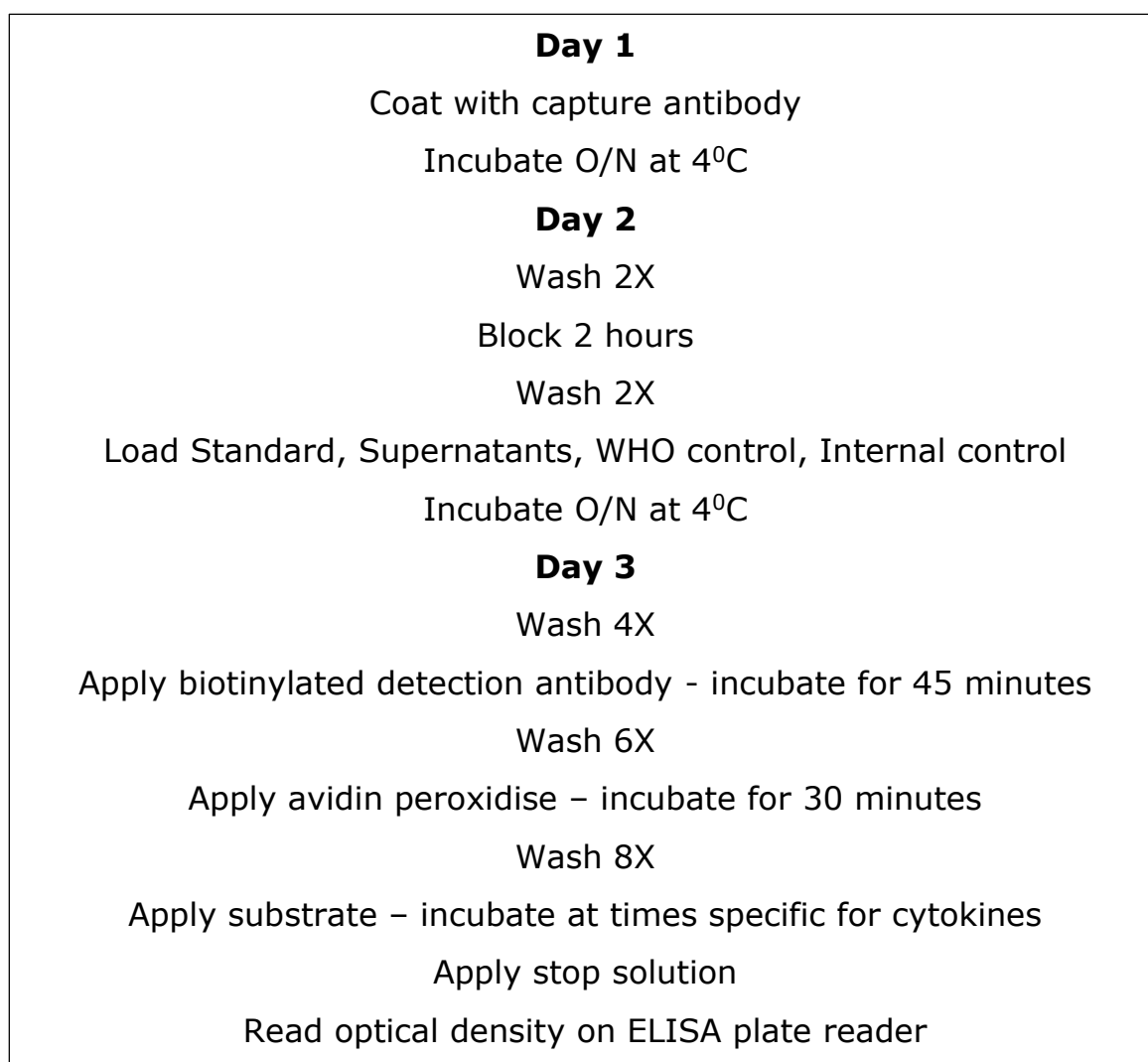


Figure 6.1: Flow chart depicting ELISA method.

6.3.3 Interpretation of ELISA results

Cytokine concentrations were calculated using ELISA software (Softmax, Molecular Devices, Menlo Park, UK) from a standard curve run in duplicate on every plate. Results were calculated from the linear part of the standard curve using a four parameter curve fit. Cytokine concentrations were reported as the concentration with the background (PBS/Tween) subtracted. Using the antibody pairs and standards as detailed, the lower and upper ranges of detection for each cytokine were as follows:

TNF α : 30 - 2000pg/ml; IL12p70: 15 - 2000pg/ml.; IFN γ : 40 - 5000pg/ml

The low cutoff value was that of the least concentrated standard and the high cutoff value was that of the second most concentrated.

For the TNF α assay, a ratio was calculated from the level obtained from stimulation with LPS + IFN γ , divided by the level obtained from stimulation with LPS alone. If this ratio was <2, the result was considered to be low. An IFN γ result of <1000 pg/ml was considered to be low. All low results were retested to eliminate laboratory error and to confirm or eliminate immunological differences between samples.

The results were accepted if the optical density read within range of the standard curve, the WHO standard external control and internal control values were within the acceptable range and the duplicates had a CV of less than 15%. If required, the ELISA was repeated on the frozen duplicate sample.

6.4 Flow Cytometry

All FACS analysis was carried out on a Beckton Dickinson FACS Calibur Flow Cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, USA) using the CellQuest analysis program.

6.4.1 Measurement of IL12R β 1

A 2 tube (unstimulated and stimulated) 2 colour (PE-FL2, APC-FL4) flow cytometric method was set up to measure IL12R β 1 expression on stimulated lymphocytes in order to quantitate differentiate receptor expression between TB patients and healthy controls. This was optimized by Dr Suzanne Anderson and myself as part of the collaborative study. A normal control was always set up for measurement of receptor expression when testing a patient.

6.4.1.1 Reagents

Fluorescently labeled antibodies obtained from Pharmingen were as follows: Anti-IL12R β 1 (PE) (Cat. No. 556065) and Anti-CD3 (APC) (Cat No 345767).

6.4.1.2 Method

PBMCs were separated from heparinized blood by Ficol according to standard procedure and the cell concentration adjusted to 1×10^6 /ml. PBMCs were pipetted into 2 Falcon 2052 polystyrene tubes for stimulated and unstimulated samples.

1ml of 1×10^6 /ml cells was added to each tube for unstimulated cells and cells stimulated with PHA, final concentration 2 μ g/ml for 72 hours. Note that both tubes are incubated at 37°C in a humidified atmosphere of 5% CO₂ for 72 hours. Tubes were centrifuged at 300g for 5 minutes and supernatant decanted leaving a residual volume of

approximately 50µl of cell suspension in the tube. 5µl IL12Rβ1 and 5µl CD3 were added to both tubes, samples mixed briefly by vortexing and incubated in the dark at RT for 30 minutes. 1 ml BD FACSTM lysing solution, diluted 1:10 in ddH₂O, was added to each tube and samples incubated for a further 30 minutes in the dark. Tubes were then centrifuged at 300g for 5 minutes. Supernatants were decanted and the pellet resuspended in 1ml sterile PBS prior to assay on a Beckton Dickinson FACS Calibur Flow Cytometer (Beckton Dickinson Immunocytometry Systems, San Gates were drawn around the specific fluorescent populations and the median fluorescent intensity (MdX) and percent fluorescence analyzed in proportion to a total of 5000 events counted. Histogram and quadrant statistics were applied.

6.5 Molecular Methods

Unless stated otherwise, all PCR primers were designed and optimized by myself.

All PCR procedures were carried out on a Perkin Elmer Applied Biosystems, GeneAmp® PCR System 9700 thermal cycler.

6.5.1 DNA extraction and quantitation

6.5.1.1 DNA extraction

Blood (2ml) was collected into EDTA tubes and DNA extracted using the QIAamp® DNA Blood Mini Kit (Qiagen Cat No 51183) according to manufacturer's instructions. In summary, 200µl of Qiagen protease, stored at 4oC was pipetted into a 15ml centrifuge tube containing 2ml of EDTA whole blood and the sample vortexed. Buffer AL (2.4ml) was added, mixed thoroughly by inverting tube 15 times, followed by additional vigorous shaking for at least 1 minute and the sample then incubated for 10 minutes in a 70°C water bath. Absolute ethanol (2ml)

was added to the sample and mixed thoroughly by inverting tube 10 times, followed by additional vigorous shaking. Half the solution was transferred onto a QIAamp® Midi column placed in a 15ml centrifuge tube and the sample centrifuged at 1850g for 3 minutes. The filtrate was discarded into bleach (Exitol) diluted to a final concentration of 10000ppm of chlorine, and the remaining solution applied to the column and centrifuged again at 1850g for 3 minutes. The filtrate was discarded into the bleach, 2ml buffer AW1 added to the Midi column and the sample centrifuged at 4500g for 1 minute. Buffer AW2 (2ml) was then loaded onto the column and the sample centrifuged at 4500g for 15 minutes. The Midi column was then placed into a clean 15ml centrifuge tube and the original tube with its contents discarded. DNA was eluted by adding 300µl TE buffer (Tris HCl 1M pH7.6, EDTA 0.5M pH8.0 in 100ml ddH₂O) directly onto the membrane of the Midi column, incubated at RT for 5 minutes and then centrifuged at 4500g for 5 minutes. Eluted DNA was stored in 1.5ml Eppendorf tubes.

6.5.1.2 DNA quantitation

Extracted DNA was quantified prior to storage and use by the PicoGreen®ds DNA Quantification Reagent (Molecular Probes, Cat No P-7581) according to manufacturer's instructions. In summary, a standard curve was prepared from calf thymus DNA (Sigma Cat No D-3663). Lyophilized calf thymus DNA was reconstituted in 2ml TE buffer giving a concentration of 1mg/ml. This was stored in aliquots at -20°C. Immediately prior to use, Picogreen Solution A was prepared by diluting 1:200 in TE buffer and Picogreen Solution B was prepared by diluting 1:380 in TE buffer. The tubes containing prepared solutions A and B were covered with aluminium foil. To prepare the STD curve 20µl of stock DNA solution was diluted with 1540µl of TE buffer in a Bijou tube (Sterilin, UK) and serial dilutions prepared in

buffer to give standards with a range of concentrations 6400ng/ml to 12.5ng/ml. Each prepared standard (50µl) was applied in duplicate across a 96 well, flat bottomed microwell plate (Nunc Cat. No. 442404) and 5µl of each DNA sample, diluted 1:10 in TE buffer, prior to assay, placed in the remaining wells. Picogreen solution A was then added to each of the STD wells to give final working concentrations of 3200ng/ml to 6.25ng/ml and 95µl of Picogreen solution B added to wells containing DNA samples, to give a final dilution of 1:200. The plate was covered with aluminium foil and incubated at RT for 5 minutes. Fluorescence was measured using an excitation wavelength of 488nm and an emission wavelength of 530nm on a Spectramax Microplate Reader (Molecular Devices). DNA concentrations were calculated relative to that of the standards using the Spectramax software, SOFTmax PRO.

6.6 DNA amplification

6.6.1 WAVE screening

6.6.1.2 PCR amplification of IL12p40 promoter for WAVE screening

Using eight overlapping pairs of primers and genomic DNA, the IL12p40 promoter fragment amplified was 2022bp long, from +73 to -1949.

The oligonucleotide primers used are listed in table 6.7.

Primer	Sequence 5' to 3'
IL12BP1F	AAGCTTCTTTTGCATAACTGGC
IL12BP1R	CAACTGGGGGAGGGGCTTT
IL12BP2F	AACTACTTCACGTCAATTTCTG
IL12BP2R	CTGGCCGTGGGTGGAGAC
IL12BP3F	AGGCCTAGAGGACACAGGG
IL12BP3R	CGCAGGTCCACTTCTCCATC
IL12BP4F	TAAAACCCGGCCTGGTCCC
IL12BP4R	GGAATTTCAAGTTGCTAAGAGG
IL12BP5F	ACATGTTCTGTTACGTGCA
IL12BP5R	GTGCTCATAGGTACTCGCTC
IL12BP6F	CTGTATGCCTCCCTGAGGG
IL12BP6R	CCTGGTTCTTCCCAAGTCAG
IL12BP7F	CCTTTGCCCTTCATCTCATCCT
IL12BP7R	TTGCAACATTGAAAACAACCTCTC
IL12BP8F	TTTTGATGTCTATGTTCCCTCC
IL12BP8R	TTGGGAAGTGCTTACCTTGCT

Table 6.7: Primers for amplification of the IL12p40 promoter for WAVE

Note: These primers were designed by Dr Suzanne Anderson as part of the Burroughs-Wellcome funded collaborative study.

PCR amplification of fragments was performed in 25 μ l volumes containing 200ng genomic DNA template, 0.5 μ M of each primer, 1.5mM MgCl₂, 200 μ M dATP, dCTP, dGTP, dTTP; 0.5 units of *Taq* DNA polymerase (Red Hot *Taq*, ABGene Cat. No. AB 0406).

Amplifications were carried out as follows: 1 cycle of 95°C for 90 seconds; 10 cycles of 95°C for 30 seconds, 56°C for 50 seconds, 72°C

for 40 seconds; 23 cycles of 95°C for 20 seconds, 56°C for 50 seconds, 72°C for 50 seconds.

6.6.1.3 Preparation of heteroduplexes

Heteroduplexes were prepared by mixing small, equal volumes of “normal” PCR product with “subject” PCR product (for example 12 μ l+12 μ l), incubating at 95°C for 5 minutes in a thermal cycler and then switching the cycler off, allowing gradual cooling for 45 minutes. This allowed the formation of homoduplexes and heteroduplexes. When running the heteroduplex samples on the WAVE instrument, a normal homoduplex sample was always loaded, in addition to the test samples and the elution patterns compared. Any pattern different from the normal sample was analyzed and considered as a possible DNA sequence change.

The melting domains of the PCR product fragments were calculated by computation prior to running the samples. If a fragment contained different melting domains, it was run through the WAVE instrument at the respective melting temperatures.

6.6.1.4 Running on WAVE instrument

Heteroduplexes were pipetted into microtubes (ABgene Cat. No. AB-0620), fitted into a 96 well plate and run on the WAVE instrument (WAVE nucleic Acid Fragment Analysis System, Model No 3500 HT, Transgenomic, Inc. Omaha, NE.) at the Department of Human Genetics, according to their procedure.

6.6.2 ARMS-PCR screening for -1523 A/G and -1564 C/T SNPs

For screening of the A/G and C/T heterozygotes, novel ARMS-PCR assays were designed, using primers IL12BP2F, IL12BP2R and an

additional forward mismatch primer, ARMS-Fg or ARMS-Ft that would amplify only the G and T alleles respectively. Amplification of the normal AA and CC genotypes resulted in 2 identical PCR fragments of 269 bp long for each of the two reactions. Amplification of the heterozygous A/G genotype resulted in 2 PCR products of 269bp and 217bp and amplification of the heterozygous C/T genotype resulted in 2 PCR products of 269bp and 176bp. The oligonucleotide primers used are listed in table 6.8.

Primer	Sequence 5' to 3' - mismatches in bold
ARMS-Fg	AATCTGCTTTGTCCACTTAATT CTG C mismatch
ARMS-Ft	CACCCCACAAATGGTATCAG GAT G mismatch
IL12BP2F	AACTACTTCACGTCAATTTCTG
IL12BP2R	CTGGCCGTGGGTGGAGAC

Table 6.8: Mismatched Allele Specific Primers for ARMS-PCR for detection of -1523 A/G and -1564 C/T SNPs

Notes:

The ARMS-Fg primer ends in G for detection of G allele

The ARMS-Ft primer ends in T for detection of T allele

The amplification conditions used were the same as for the WAVE screening, except for the primer concentrations, where 0.3 μ M of IL12BP2F, 0.8 μ M of ARMS-Fg, 0.8 μ M of ARMS-Ft and 0.6 μ M of IL12BP2R were used and Go Taq[®] polymerase (Promega Cat No M3001) was used.

Amplifications were carried out as follows: 1 cycle of 94°C for 2 minutes; 33 cycles of 94°C for 15 seconds, 59°C for 30 seconds, 72°C for 2 minutes; 1 cycle of 72°C for 7 minutes, 25°C for 2 minutes.

6.7 Cloning

6.7.1 PCR Amplification of the IL12p40 promoter for cloning

The original oligonucleotides used in WAVE screening were adapted as shown in table 6.9, resulting in a PCR product of 2031bp.

Primer	Sequence with 9bp 5' to 3' extensions	Incorporated restriction site
Original P1F	AAGCTTCTTTTGCATAACTGGC	
Adapted P1F	ACAGAGCTCCTTTTGCATAACTGGC	GAGCT'C <i>SacI</i>
Original P8R	TTGGGAAGTGCTTACCTTGCT	
Adapter P8R	ATACTCGAGGGAAGTGCTTACCTTGCT	C'TCGAG <i>XhoI</i>

Table 6.9: Uniquely adapted primers for cloning

PCR amplification of fragments was performed in 25µl volumes containing 200ng genomic DNA template; 0.5µM of each primer; 1.5mM MgCl₂; 200µM dATP, dCTP, dGTP, dTTP; 0.5 units of Expand Hi Fidelity PCR system *Taq* polymerase (Roche Cat No 011 504 4600).

Amplifications were carried out as follows: 1 cycle of 94°C for 2 minutes; 33 cycles of 94°C for 15 seconds, 59°C for 30 seconds, 72°C for 2 minutes; 1 cycle of 72°C for 7 minutes, 25°C for 2 minutes.

For this amplification, using the Expand *Taq* polymerase, the elongation time was extended according the manufacturer's instructions.

6.7.2 Purification of PCR product

Several tubes of PCR product of each of the fragments to be cloned were pooled respectively and cleaned up using a QIAquick® PCR purification kit (Qiagen Cat No 28104). All centrifugations were carried out at 13000 rpm. In summary, 5 X volume of lysis buffer PB

was added to PCR product and mixed by vortexing. Each sample was applied to a spin column placed in a collection tube, centrifuged for one minute, the flow-through discarded and the column placed back into the collection tube. For the washing step, 750µl of buffer PE was added to the columns which were centrifuged for 1 minute, the flow-through discarded and the column placed back in to the collection tube and samples centrifuged for an additional minute. For the elution step, the columns were placed in sterile 1.5ml Eppendorf tubes, 30µl of buffer EB added to the centre of each column, left to stand for one minute and then centrifuged for one minute.

6.7.3 Cloning into T vector system

The purified PCR products (normal promoter, SNP -1523 G and SNP -1564 T promoter fragments) were cloned into a T vector, using the pGEM®-T Easy Vector System II (Promega A1380). The purified insert DNA was ligated into the vector at 4°C overnight, using a 1:1 molar ratio of vector:insert. A positive control and background control were included. A transformation control, using uncut plasmid, was not done as the T vector was supplied linearized. Transformation was done by adding 2µl of each ligation reaction to 50µl JM109 competent cells, leaving on ice for 20 minutes, heat shocking at 42°C for 50 seconds, again leaving on ice for two minutes, adding 950µl of SOC medium kept at RT and then incubating shaking at 37°C for 1.5 hours. Then 20µl of each transformation culture was plated onto duplicate Luria Bertoni (LB) plates containing 100µg/ml ampicillin, 0.5mM IPTG and 80µg/ml X-Gal and incubated overnight at 37°C. After this incubation, the white colonies were screened for inserts by PCR. A blue colony was screened as a negative control.

6.7.3.1 Ligation of normal, SNP -1523 G and SNP -1564 T promoter fragments into T vector system

The ligation mix used for ligating the three promoter fragments into the T vector are shown in table 6.10.

2X rapid ligation buffer	5 μ l
pGEM®T easy vector, linearized, cleaned	1 μ l (50ng)
PCR product, digested, cleaned	1 μ l (50ng)
T4 DNA ligase	1 μ l
Sterile water	2 μ l
Total volume	10 μ l

Table 6.10: Ligation mix used for cloning into T vector system

6.7.3.2 Transformation of competent cells

LB agar plates were prepared by dissolving 2.5g bactotryptone, 1.25g yeast extract, 1.25g sodium chloride and 3.75g agar in 250ml water and autoclaving. The agar was allowed to cool to 55°C and 250 μ l of 100mg/ml ampicillin added, giving a final concentration of 100 μ g/ml ampicillin, poured into sterile petri dishes and allowed to set. The plates were checked for sterility by incubating at 37°C overnight.

Onto each LB ampicillin plate was spread 100 μ l of 100mM IPTG and 20 μ l of 50mg/ml Xgal. This was allowed to absorb onto the plates at 37°C for 30 minutes.

Working on ice, 2 μ l of each ligation reaction was added to a sterile tube and 50 μ l competent cells, just thawed, added.

For a transformation control, uncut plasmid is required. This control was not done as the vector from the kit was supplied linearized.

A background control was done by transforming with linearized vector without an insert

The tubes were kept on ice for 20 minutes, heat shocked at 42°C in a waterbath for 45-50 seconds, kept on ice for two minutes and then 950µl SOC medium equilibrated to room temperature added.

The SOC medium was prepared by dissolving 1g bactotryptone, 0.25g yeast extract, 0.5ml 1M NaCl and 0.125ml KCl in 47ml distilled H₂O, autoclaving and then adding 0.5ml filter sterilized 2M Mg²⁺ and 0.5ml filter sterilized 2M glucose. The volume was then made up to 50ml, pH checked (required to be 7.0), aliquoted and stored at -80°C.

The tubes were incubated at 37°C for 1.5 hours with shaking.

A volume of 25µl from each tube was plated onto two LB/ampicillin/IPTG/Xgal plates and incubated at 37°C overnight.

6.7.3.3 Screening of colonies for inserts

For the screening, a small part of the culture was scraped with a sterile tip and placed into 50µl sterile water. This bacterial suspension was heated to 95°C for 5 minutes to destroy DNAses and then 10µl used for the PCR reaction. The internal primers designed for the WAVE technique were used for this screening.

The following morning white colonies were then picked off the plates with sterile tips, master plates prepared and incubated at 37°C overnight. Colonies that remained white were dipped into volumes of 50µl sterile water in tubes, heated to 95°C for 5 minutes to degrade DNAses and then placed on ice. PCR as previously described using any pair of the WAVE primers was done on 10µl of the water containing

heat treated colony. The PCR product was run on a 1% agarose gel for visualization for confirmation of insert in colony.

6.7.3.4 Sequencing

The sequencing reaction was carried out using the Big Dye® Terminator Cycle Sequencing kit (Applied Biosystems Cat No 4336915) by PCR.

PCR amplification of cloned promoter fragments was performed in 20ul volumes containing 3µl miniprep DNA (300ng); 10µl water; 3.3µM of primer (1µl) [10µM diluted 1:3]); 2µl Big Dye Terminator Mix; 4µl 5X buffer.

Amplification was carried out as follows: 1 cycle of 96°C for 10 seconds; 25 cycles of 96°C for 10 seconds, 52°C for 15 seconds, 60°C for 4 minutes; 1 cycle of 60°C for 4 minutes, 4°C till taken out of machine.

6.7.3.5 Overnight cultures, freezing and storage of colonies with inserts

Single colonies were scraped from the master plates into 5ml LB medium with 5ul of 100mg/ml ampicillin, giving a final concentration of 100µg/ml ampicillin and incubated at 37°C overnight with shaking.

The following morning 2 tubes each of 1ml culture was mixed with 1ml freezing solution, snap frozen in liquid nitrogen and stored at -70°C for future resources of material.

A 1ml volume of saturated culture from a 16 hour culture was added to 1ml glycerol solution in a cryostat vial, snap frozen in liquid N₂ and stored at -70°C. When it was necessary to reculture the clone, the culture was revived by scraping frozen splinters onto an agar plate.

The glycerol stock was never thawed completely as freeze/thaw cycles reduce viability.

6.7.3.6 Plasmid miniprep DNA extraction

The plasmid DNA was isolated using the Qiaprep®Spin Miniprep Kit (Qiagen Cat No 27104). All centrifugations were carried out at 13000rpm in a microcentrifuge. In summary, overnight cultures were done by incubating at 37°C and shaking at 150rpm, 4ml volumes of LB medium containing 100ug/ml ampicillin and a single colony. Bacterial cells were harvested by centrifugation. Supernatant was taken off, the pelleted bacterial cells resuspended in 250µl Buffer P1 and transferred to a microfuge tube. A 250µl volume of buffer P2 was added, the tube mixed by inverting, 350µl buffer N3 added and the tube again mixed by inverting. The tube was centrifuged for 10 minutes and the supernatant applied to a spin column. The spin column was centrifuged for 1 minute, flow through discarded, 500µl wash buffer PB added, centrifuged for 1 minute, flow through discarded, 750µl PE added, centrifuged for 1 minute, flow through discarded and centrifuged for an additional minute. The plasmid DNA was then eluted by adding 50µl buffer PE, allowed to stand for 1 minute and then centrifuged for 1 minute.

6.7.4 Cloning of promoter fragments into pGL3-Basic vector

6.7.4.1 Linearization of pGL3-Basic vector by double digestion

As preparation for the ligation reaction, the pGL3-Basic was double digested to make the restriction sites in the multiple cloning site available for the inserts to be cloned.

The digestion mix used for linearization of the pGL3-Basic vector is shown in table 6.11.

pGL3-Basic vector	2 μ l (1200ng)
<i>Xho</i> I	2.0 μ l
10 X Buffer D	1.0 μ l
BSA 10mg/ml	1.0 μ l
Casein 10mg/ml	1.0 μ l
Sterile water	3.0 μ l
Total Volume	10.0 μ l
Incubation	37°C for 90 mins

Followed by

<i>Sac</i> I	2.0 μ l
10 X Buffer J	2.0 μ l
10 X Buffer D	2.0 μ l
Sterile water	4.0 μ l
Total Volume	20.0 μ l
Incubation	37°C for 90 mins

Table 6.11: Double digestion mix for linearization of pGL3-Basic vector

6.7.4.2 Release of promoter fragments from T vector by double digestion

As preparation for the ligation reaction, the normal and SNP -1564 T promoter fragments were double digested, releasing them from the T vector and making the *Xho*I and *Sac*I restriction sites at their ends available.

The digestion mix used for double digestion of the promoter fragments is shown in table 6.12.

Plasmid DNA miniprep	8 μ l (100ng)
<i>Xho</i> I	1.0 μ l
10 X Buffer D	1.5 μ l
BSA 10mg/ml	1.5 μ l
Casein 10mg/ml	1.0 μ l
Sterile water	2.0 μ l
Total Volume	15.0 μ l
Incubation	37°C for 90 mins

Followed by

<i>Sac</i> I	1.0 μ l
10 X Buffer J	2.0 μ l
10 X Buffer D	1.5 μ l
Sterile water	1.5 μ l
Total Volume	20.0 μ l
Incubation	37°C for 90 mins

Table 6.12: Digestion mix used for releasing normal and SNP - 1564 T fragments from T vector

6.7.4.3 Gel extraction of DNA fragments

In order to ensure integrity of the DNA, 40% sucrose was used as loading buffer for electrophoresis and fragments viewed under long wave UV light.

After electrophoresis and viewing, the appropriate fragments were excised out of the gel.

The DNA was extracted from the agarose using the QIAEX II Gel Extraction kit (Qiagen Cat No 20021).

In summary, the following procedure was carried out.

All centrifugations were carried out at 13000 rpm.

Gel slices were weighed, 3 X volume of QIAEXI buffer added and vortexed for 30 seconds. The QIAEXII beads were resuspended by vortexing and 30 μ l added to each gel slice in QIAEXI buffer. The gel slices were incubated at 50°C for 10 minutes, vortexing every 2 minutes to solubilize the agarose and bind DNA to the beads. Samples were centrifuged for 30 seconds, supernatants removed, pellets washed once in 500 μ l QIAEXI buffer and twice in 500 μ l PE buffer. Pellets were air dried for at least 30 minutes till white. DNA was eluted by adding 20 μ l sterile water, resuspended by vortexing, incubated at room temperature for 5 minutes, centrifuged and eluate taken off into a sterile Eppendorf tube. The elution procedure was repeated one more time.

6.7.4.4 Creation of SNP -1523 G promoter fragment

6.7.4.4.1 Digestion of AG4 and AG44 clones with *Sma*I for creation of the SNP -1523 G fragment.

The digestion mixes used for creating correct fragments from AG4 and AG44 clones are shown in table 6.13. Separate digestion mixes were set up for each of the AG4 and AG44 clones.

AG4 and AG44	60 μ l (total ng extracted from gel)
<i>Sma</i> I	2.0 μ l
10 X Buffer J	8.0 μ l
Sterile water	2.0 μ l
Total Volume	80.0 μ l
Incubation	25°C for 60 mins

Table 6.13: Digestion mix for creation of fragments for the SNP -1523 G promoter fragment

6.7.4.4.2 Ligation of AG4 and AG44 fragments for creation of SNP -1523 G fragment and cloning into pGL3-Basic vector in single step

The ligation mix for creation and cloning of mutant -1523 G fragment into pGL3-Basic vector is shown in table 6.14.

2 X ligation buffer	8 μ l
pGL3-Basic linearized, cleaned	1.0 μ l (50ng)
AG44 (663bp fragment)	3.0 μ l (20ng)
AG4 (1359bp fragment)	1.0 μ l (20ng)
*T4 DNA ligase	3 μ l
Total volume	16 μ l

Table 6.14: Ligation mix for creation and cloning of fragments for SNP -1523 G into pGL3-Basic vector

*Additional T4 DNA ligase was used in this reaction, as two fragments were ligated together.

6.7.4.5 Creation of truncated promoter fragment

6.7.4.5.1 PCR amplification for creation of truncated promoter

Uniquely adapted primers shown in table 6.15 were designed, resulting in a truncated promoter fragment of 1744bp in length.

Primer	Sequence 9bp 5' extension	Incorporated restriction site
Adapted P1F	ACAGAGCTCCTTTTGCATAACTGGC	GAGCT'C <i>SacI</i>
Adapted R truncated	ATACTCGAGGGTTTACATCATGGCCA	C'TCGAG <i>XhoI</i>

Table 6.15: Uniquely adapted primers for creation of truncated promoter

PCR conditions were as previously described for WAVE screening.

6.7.4.5.2 Ligation mix for cloning truncated construct into pGL3-Basic vector

The ligation mix for cloning the truncated promoter fragment into the pGL3-Basic vector is shown in table 6.16.

2 X rapid ligation buffer	5µl
pGL3-Basic linearized, cleaned	1µl (50ng)
PCR product digested, cleaned	3µl (50ng)
T4 DNA ligase	1µl
Total volume	10µl

Table 6.16: Ligation mix for cloning of truncated promoter into pGL3-Basic

6.7.4.6 Creation of deletion promoter construct

6.7.4.6.1 PCR amplification of fragments 1 and 2 for creation of deletion construct

Uniquely designed primers used for generation of fragments 1 and 2 for creation of the deletion construct are shown in tables 6.17 and 6.18.

Primer	Sequence 9bp 5' extensions	Incorporated restriction site
Adapted P1F	AC AGAGCTCCTTTTGCATAACTGGC	GAGCT'C <i>Sac</i> I
Adapted 8R	AT ACTCGAGGGGAAGTGCTTACCTTGCT	C'TCGAG <i>Xho</i> I

Table 6.17: Primers used for generation of full length promoter -1952 to +79 (to be digested with *Ba*/I), creating 1673bp of deletion construct

Primer	Sequence with 9bp 5' extension and new forward primer	Incorporated restriction site
New F	GAGAGTTGTTTTCAATGTTGCAACAAGTC	
Adapted 8R	AT ACTCGAGGGGAAGTGCTTACCTTGCT	C'TCGAG <i>Xho</i> I

Table 6.18: Primers used for creation of the -94 to +79 promoter region creating 173bp fragment of deletion construct

6.7.4.6.2 Digestion of full length fragment for creation of deletion construct

The digestion mix used for fragment 1 is shown in table 6.19.

Eluate of normal construct	40 μ l
<i>Ba</i> l	2.0 μ l
10 X Buffer G	6.0 μ l
BSA 10mg/ml	6.0 μ l
Sterile water	6.0 μ l
Total Volume	60.0 μ l
Incubation	37°C for 60 mins

Table 6.19: Digestion mix for fragment 1

6.7.4.7 Ligation and cloning of deletion construct into pGL3-Basic vector in single step

The ligation mix used for ligating of 1675bp and 173bp fragments and cloning into the pGL3-Basic vector in a single step is shown in table 6.20.

2 X rapid ligation buffer	8 μ l
pGL3-Basic linearized, cleaned	1 μ l (50ng)
Digested, large fragment (1675bp)	2 μ l (50ng)
Small fragment (173bp)	2 μ l (50ng)
*T4 DNA ligase	3 μ l
Total volume	16 μ l

Table 6.20: Ligation mix for cloning of 1675bp and 173bp fragments into the pGL3-Basic vector in a single step

*Additional T4 DNA ligase was used in this reaction, as two fragments were ligated together.

6.8 Visualization of PCR products by agarose gel electrophoresis

10 μ l of the PCR reactions was mixed with 2 μ l of 6 X DNA loading buffer and electrophoresed in 1 X TAE buffer, at 80V on an agarose gel prepared in 50ml of 1 X TAE containing 2 μ l of 10mg/ml ethidium

bromide. For visualization of the PCR products a 1 or 2% agarose gel was used. For visualization of the ARMS-PCR products, a 3% gel was used for separation of the two bands similar in size.

An appropriate molecular weight marker was run on the gel for comparison with the PCR product. DNA was visualized using a transilluminator.

When the Go *Taq*[®] polymerase was used, the green reaction buffer used in the PCR reaction, had a dual purpose, being the loading buffer as well. For samples amplified using this system, the PCR product was loaded directly onto the gel.

6.9 Dual Luciferase Reporter Assay

6.9.1 Optimization

Optimizing experiments were done as follows:

Transfection experiments were done using the mouse macrophage RAW 264.7 cell line, the human monocytic THP-1 cell line and the human HT1080 cell line.

Two internal controls pRL-TK (50ng) and pRL-CMV (10ng) were used.

Three transfection reagents, FuGENE[®] HD Transfection Reagent (Roche Cat. No. 04709713001), X-tremeGene HP DNA Transfection Reagent (Roche Cat. No. 06365752001) and X-tremeGene 9 DNA Transfection Reagent (Roche Cat. No. 0636651100) were used.

Total incubation times of 34 and 48 hours were used.

After optimization experiments, X-tremeGene HP DNA Transfection Reagent was used to transfect the mouse macrophage RAW 264.7 cells, with internal control pRLTK in a total incubation time of 48 hours.

6.9.2 Method

The Dual Luciferase Reporter Assay was a four day assay. In summary the following procedure was carried out.

Day 1

Cells were plated into a 12 well tissue culture plate. Cells grown in complete medium (RPMI + 10% FCS) in at atmosphere of 5% CO₂, passaged every 2 days were used. These were harvested from two 10cm tissue culture dishes by washing with complete medium. The cell suspension was collected into a 50ml tube and centrifuged at 10000 rpm for 3 minutes and the cell pellet resuspended in 10ml complete medium. A cell count was done by loading 10ul of the cell suspension onto a counting chamber, 4 squares counted and the mean calculated. The cell concentration was adjusted to 6×10^4 cells/ml. Cells (1ml) at a concentration of 6×10^4 cells/ml were pipetted into each well of the 12 well plate and grown overnight to a confluency of 60-70%.

Day 2

Transfection was done on day 2. First the DNA to be transfected was prepared. Five constructs, the normal promoter, SNP -1523 G and SNP -1564 T promoters, a truncated promoter and promoter with essential elements deleted, all in the pGL3-Basic vector were used. Miniprep plasmid DNA extractions of these promoter constructs in the pGL3-Basic vector had been previously prepared and stocks of known

concentration frozen. 500ng DNA of the cloned normal, SNP -1523 G, SNP -1564 T, truncated and deletion promoters, as well as the empty pGL3 vector were mixed with 10ng of pRL-TK, the internal control Renilla reniformis Luciferase Reporter vector. These samples were prepared in duplicate in 12 Eppendorf tubes. Serum free medium was prewarmed to room temperature and transfection reagent equilibrated to room temperature and mixed well before use.

Using FuGENE®HD transfection reagent, for each transfection, 48µl serum-free medium and 2µl FugGENE were added to an Eppendorf tube. FuGENE must be added directly into medium and not come into contact with the plastic tube. This was incubated at room temperature for 15 minutes.

A master mix of this was prepared as follows:

13 X 48µl = 624µl medium

13 X 2µl = 26µl FuGENE

After incubation at room temperature, 50µl of this master mix was added to each Eppendorf tube containing the DNA transfected (500ng plasmid DNA + 10ng pRL-CMV). Duplicates for each reaction were prepared. The DNA with FuGENE mixes were mixed and incubated at room temperature for 15 minutes.

Using the X-tremeGENE and X-tremeGENE 9 DNA transfection reagents, the DNA mixes were diluted to a minimum volume of 100µl with serum free RPMI and the transfection reagent was added directly to the tubes containing DNA.

4:1 ratio of transfection reagent:µg DNA was used (4.2µl XtremeGENE added to 1050µg DNA [1000ng + 50ng])

A transfection control, kindly donated by the South African Aids Vaccine Institute laboratory, IIDMM, was included in each experiment. The DNA mix for this was 1000ng of normal promoter + 100ng LucZ/SV40 in pGL4 basic vector.

The transfection Reagent/DNA complex was added dropwise onto the cells in a 12 well plate and immediately mixed gently to ensure equal distribution over entire plate surface. The plate was incubated at 37°C in 5% CO₂. Thirteen or 16 hours after transfection, 1.2% DMSO was added to each well (12µl to 1000µl) except the unstimulated well. Five or 16 hours later a final concentration of 1µg/ml IFN γ was added to each well (50µl of 20µg/ml) except the unstimulated well. Eight hours later a final concentration of 1µg/ml LPS was added to each well (100µl of 10µg/ml) except the unstimulated well. Eight hours later, cells were harvested.

Day 3

On day 3, cells were harvested as follows: passive lysis buffer was equilibrated to room temperature. The plate was centrifuged at 3000rpm for 5 minutes, medium was gently taken off all wells and cells washed 2 X with 1ml of sterile PBS with a sterile squeeze pipette, centrifuging as before after each wash. After the PBS had been removed, 100µl of passive lysis buffer was added to each well and the plate shaken on a plate shaker for 15 minutes at room temperature. All the cell lysate from each well was taken into an Eppendorf tube and stored at -80oC for one hour or overnight.

The following time sequences were carried out:

Transfection → 16 hours, 1.2% DMSO added → 16 hours, IFN γ 1 μ g/ml added → 8 hours, LPS 1 μ g/ml added → 8 hours, cells harvested. Total incubation time 48 hours.

Transfection → 13 hours, 1.2% DMSO added → 5 hours, IFN γ 1 μ g/ml added → 8 hours, LPS 1 μ g/ml added → 8 hours, cells harvested. Total incubation time 34 hours (Optimization).

Day 4

The Relative Light Units (RLUs) of the cell lysates were read on the luminometer (Luminoskan Ascent luminimeter (Thermo Labsystems). Eppendorf tubes containing cell lysates were centrifuged at 10000 rpm for 1 minute and the supernatant taken off into clean tubes, leaving a pellet of cell debris behind. Four samples were read on the luminometer at a time. 10 μ l of each sample was pipetted into a 96 well plate and 50 μ l of LARII reagent added. On addition of the LARII reagent, the firefly luciferase activity was measured. An aliquot (50 μ l) of the STOP & GLO[®] Reagent (50 X substrate diluted in buffer) was added to each sample in well. On addition of the Stop & Glo[®] Reagent, the firefly luciferase activity was quenched and the Renilla reniformis luciferase activity (internal control) measured.

6.10 Statistical Analysis

Statistica 9 and GraphPad Prism 6 were used for statistical analysis.

CHAPTER 7: Final Discussion

Expansion of our understanding of the IL12/IL23/IFN γ pathway came about with the identification of mutations in the IFN γ R1, IFN γ R2, IL12R β 1, IL12p40 and STAT1 genes. This resulted in a new area of research and clinical activity, adding to the understanding of mechanisms underlying human susceptibility to mycobacterial disease. Individuals with MSMD, caused by mutations in these genes, are usually affected with infections caused by poorly pathogenic mycobacteria and, less commonly, *Salmonella*. However, following the initial description of these mutations, a subset of these patients were found to be infected with *M. tb* (Altare *et al.* 2001, Caragol *et al.* 2003, Ozbek *et al.* 2005, Tabarsi *et al.* 2011). This raises the possibility that susceptibility to *M. tb* in the general population is a consequence of single gene defects in the IL12/IL23/IFN γ pathway and that TB in the general population is a Mendelian disorder (Alcais 2005).

This work was part of a collaborative study that screened a well-defined cohort of paediatric TB cases and healthy controls from the Western Cape region of South Africa, a TB-endemic area, for mutations in genes of the IL12/IL23/IFN γ pathway, associated with TB. When the study started, the IL12p40 promoter had not been extensively screened for mutations causing susceptibility to TB. The gene is a good candidate for TB susceptibility, since IL12 is important for IFN γ signalling and the clinical phenotype of subjects with defects in the gene is relatively mild, with affected individuals surviving into adult life. There was therefore, a high likelihood of finding functional polymorphisms within the promoter region of the gene that were likely to affect disease susceptibility

The majority of published TB susceptibility studies have been carried out on adults since confirming disease with the gold standard of mycobacterial culture is more easily achieved in adults than children. However, paediatric studies are important since the mechanisms of susceptibility are likely to differ from adults, as children tend to develop primary progressive TB disease rather than reactivation of dormant infection that classically characterises adult disease.

To investigate the role of gene defects in the IL12p40 promoter in TB susceptibility, a subset of the main paediatric cohort (total n=144, cases n=94, controls n=50) was first screened for DNA changes by the WAVE technique. Two heterozygous SNPs -1523 A/G (total n=6, cases n=1, controls n=5) and -1564 C/T (total n=8, cases n=5, controls n=3) were identified. Although numbers were small, analysis showed the -1523 A/G SNP to be more prevalent in controls (p=0.02) suggesting that it may have a role in protection from TB. The observation that the -1523 A/G SNP occurred within a genetic sequence (GT**A**TA), reported to be important for nuclear binding, also suggested that it may have a functional effect on IL12p40 production.

In order to evaluate the role of these SNPs, further screening of the whole paediatric cohort was then done. A cohort of healthy adult controls, from a TB-endemic area, none of whom had a history of TB disease, but had been exposed to TB, was also included to increase the numbers to add power to the analysis. For the additional screening, a unique ARMS-PCR system with specifically designed mismatch primers was set up, a more cost effective method. The additional screening of the larger cohort (total paediatric samples n=397, cases n=252, healthy controls n=145; total adult healthy controls n=756), increased the numbers with the -1523 A/G (total

n=61, cases n=12, controls n=49), and -1564 C/T (total n=60, cases n=20, controls n=40) SNPs. Analysis showed that the -1564 C/T SNP may contribute to susceptibility to TB disease ($p=0.03$).

Functional testing of these two SNPs was done, using a reporter assay. In preparation for this, uniquely adapted primers that included restriction sites corresponding to those in the multiple cloning site of the expression vector were designed. The normal and two promoter fragments with the SNPs were PCR amplified with the adapted primers, facilitating cloning into the expression vector. In addition, a truncated promoter and one with essential regions deleted, were created as negative controls. The five promoter fragments were cloned into the expression vector and functional differences measured by reporter assay. An increase was seen in stimulated promoter fragments over the deletion control, but no marked functional differences between the normal and variant promoter fragments were observed. Reasons for this may have been a lack of sensitivity of the reporter assay for detecting subtle differences.

Functional cytokine studies on patients with MSMD have demonstrated distinct immunological phenotypes, the type and severity of which correlate with gene defect. To determine if patients with TB had a distinct immunological phenotype compared to healthy controls, immune testing was done by measurement of cytokines $\text{TNF}\alpha$, IL12p70 and $\text{IFN}\gamma$ after stimulation in WBAs and measurement of IL12R β 1 by flow cytometry. A very heterogeneous pattern of cytokine responses was seen.

The $\text{TNF}\alpha$ production in response to LPS with and without the addition of $\text{IFN}\gamma$ showed ratios to be lower in TB cases than the healthy controls ($p<0.0001$), probably due to an enhanced inflammatory response to

LPS in both Active and Past History TB cases with high levels of $\text{TNF}\alpha$ to LPS alone.

IL12p70 levels were also lower in the cases compared to healthy controls ($p < 0.0001$), likely due to the clinical disease.

Responses to $\text{IFN}\gamma$ were varied, with responses to PHA higher in Active TB cases, compared to healthy controls ($p = 0.002$), probably to an enhanced immune response in the disease state. $\text{IFN}\gamma$ levels in response to PPD however, were seen to be higher in healthy controls, compared to TB cases. This is probably due to the fact that most $\text{IFN}\gamma$ producing cells are located at the site of active disease, a feature exploited by some researchers in developing TB diagnostic tests (Sutherland *et al.* 2012).

Analysis of IL12R β 1 expression showed no individuals with absent receptor expression. However, there was a clear bimodal distribution of responses with a group of TB cases having low expression compared with controls. It was not possible to determine if these individuals showed a reduced ability to reponse to IL12.

The functional role of the SNPs was investigated by comparing cytokine responses in the different genotypes. No differences were seen between wildtype and SNP genotypes in IL12 levels.

Analysis of $\text{IFN}\gamma$ responses to PHA showed no differences between genotypes for the -1523 A/G SNP.

For the -1564 C/T SNP, $\text{IFN}\gamma$ levels in response to PHA showed wildtype TB cases to have higher levels than healthy controls with SNP genotype ($p = 0.01$). TB cases with SNP genotype were seen to have lower $\text{IFN}\gamma$ levels in response to PPD than wildtype healthy controls

($p=0.002$). Healthy controls with -1564 C/T genotype were found to have lower levels of $\text{IFN}\gamma$ in response to PPD than the wildtype TB cases ($p=0.0005$).

It is highly likely that the heterogeneous results identified in this work are due to the very small numbers of individuals with SNP genotype. As a result, prediction of immune phenotype and correlation with the genotype could not be done. For accurate statistical analysis, greater numbers of cases should be screened to increase the frequency of SNPs thereby adding power to the statistical analysis.

One of the challenges of doing studies on children is recruiting enough numbers of TB cases with culture proven disease. If a study like this were to be repeated, it would be possible to increase numbers by doing a multi-site study. Sample size analysis should be used in order to calculate numbers for sufficient power for statistical analysis.

There were a number of limitations to the work in this thesis. First, because only a small sub-group of paediatric TB cases were screened by the WAVE technique it is possible that some additional promoter mutations were missed. Had an enriched cohort been used, including patients with more severe clinical phenotypes (*eg* TB meningitis, miliary TB) in the initial WAVE screening, more mutations may have been identified. However, at the time of completing this work the cost of doing the WAVE technique prohibited the screening of more samples.

Second this cohort was genetically heterogeneous - a reflection on the mixed population that accessed the health services at RCWMCH and surrounding clinics. Due to the small numbers recruited overall, a decision was made not to stratify by ethnicity group and it is possible

that this will have further weakened the power to detect significant differences in both phenotype and SNP genotype between cases and controls.

Third, because both TB infection and disease are on a continuous spectrum, it is possible that some of the tuberculin positive paediatric controls could themselves have become cases over time. Similarly, because not all the TB cases had culture proven disease, but were diagnosed on the clinical likelihood of disease, there exists the possibility that some may have had another disease altogether and been incorrectly categorised as TB cases.

Predisposition to TB is complex and while single gene mutations may play a role in disease susceptibility, it is likely that in the general population there are small effects from multiple interacting genes of the IL12/IL23/IFN γ pathway that have an effect. Complex combinations of SNPs on many genes may have associations.

The methodology used for this thesis, although scientifically sound, is now outdated, but could still be used for small scale screening. During the extended time frame over which the work was done, methodology has rapidly expanded and advanced. Today, screening can be done on a much larger scale, using techniques such as whole genome sequencing, with high throughput SNP platforms. This way, associations between different SNPs in known candidate genes as well as novel association with, as yet, unidentified genes can be detected.

Today many studies are also being done using gene expression patterns for identification of biomarkers (Mistry *et al.* 2007, Jacobsen *et al.* 2007, Berry *et al.* 2010, Lesho *et al.* 2011, Lu *et al.* 2011, Wang *et al.* 2011, Maertzdorf *et al.* 2012, Ottenhof *et al.* 2012, Kaforou *et al.*

2013, Anderson *et al.* 2014). This methodology has the potential to distinguish between clinical TB groups such as active and latent disease and can identify subjects who are at risk of recurrent disease, as shown in a study by completed on South African adults (Mistry *et al.* 2007). Other studies have shown it to be a powerful tool for discriminating between active TB, latent TB and uninfected individuals (Lu *et al.* 2011, Maertzdorf *et al.* 2012).

Most of these studies have been done on adults, but a study done on a Warao Amerindian paediatric cohort identified a predictive 116 gene signature for discriminating active from latent TB disease (Verhagen *et al.* 2013). Data from this study suggests that this signature be used as a biomarker for potential childhood TB diagnosis. A recent study on childhood tuberculosis in Africa has identified tuberculosis-specific transcriptome signatures, capable of distinguishing tuberculosis from latent tuberculosis and other common infectious diseases that may mimic tuberculosis. Furthermore this study explored the diagnostic potential of these signatures and strongly suggested that gene expression signatures may have a future role in the diagnosis of childhood tuberculosis (Anderson *et al.* 2014).

It would be informative to know which genetic defects may be associated with TB. Today, rather than screening for single gene defects by association studies, whole genome sequencing may be performed to evaluate the effect of multiple gene polymorphisms and / or mutations on disease susceptibility to disease. Furthermore, genetic expression studies are starting to yield insights about the pathogenesis of TB and the study of genetic polymorphisms in the context of these gene expression patterns has potential for providing greater understanding in this field. For example, in the recent study by

Berry, *et al.* 2010 showed that the TB signature identified in their study was dominated by a neutrophil-driven interferon inducible gene profile, suggesting that Type I IFN $\alpha\beta$ signalling may play an important role in TB pathogenesis.

In conclusion, in this study two previously unreported polymorphisms within the IL12p40 promoter have been identified and documented in the SNP database for further studies. However, no significant differences were found between cases and controls for these variants and furthermore, it was not possible to demonstrate any functional effects of these sequence variants. Thus, the IL12p40 promoter appears to be highly conserved and mutations are extremely rare.

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